PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [6] : **C12N 9/00**	**A2**	(11) International Publication Number: **WO 98/37181** (43) International Publication Date: 27 August 1998 (27.08.98)

(21) International Application Number: PCT/US98/03404

(22) International Filing Date: 20 February 1998 (20.02.98)

(30) Priority Data:
60/038,750	20 February 1997 (20.02.97)	US
60/047,151	20 May 1997 (20.05.97)	US
60/054,549	1 August 1997 (01.08.97)	US
60/055,762	14 August 1997 (14.08.97)	US
60/064,322	30 October 1997 (30.10.97)	US

(71) Applicant (for all designated States except US): WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH [US/US]; Nine Cambridge Center, Cambridge, MA 02142 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): COUNTER, Christopher, M. [CA/US]; Apartment 5R, 310 Prospect Street, Cambridge, MA 02139 (US). MEYERSON, Matthew [US/US]; 11 Aqueduct Road, Wayland, MA 01778 (US). WEINBERG, Robert, A. [US/US]; 25 Copley Street, Brookline, MA 02146 (US).

(74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02173 (US).

(81) Designated States: CA, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

(54) Title: TELOMERASE CATALYTIC SUBUNIT GENE AND ENCODED PROTEIN

(57) Abstract

Isolated DNA encoding the catalytic subunit of a eukaryotic telomerase holoenzyme, such as the catalytic subunit of a yeast or human telomerase holoenzyme; the RNA transcript, which is expressed in primary human tumors, cancer cell lines and telomerase–positive tissues; and the encoded catalytic subunit protein. Methods of assessing cells for malignancy or an increased likelihood of progression to malignancy and methods of diagnosing or aiding in the diagnosis of development of malignancy in an individual are also described.

-1-

TELOMERASE CATALYTIC SUBUNIT GENE AND
ENCODED PROTEIN

RELATED APPLICATIONS

This application claims the benefit of U.S.
10 Provisional Application No. 60/038,750, filed February 20,
1997, entitled "Identification of a Protein Subunit of the
Yeast Telomerase Holoenzyme" by Christopher M. Counter,
Matthew Meyerson and Robert A. Weinberg; of U.S.
Provisional Application No. 60/047,151, filed May 20, 1997,
15 entitled "Human Telomerase Catalytic Subunit" by
Christopher M. Counter and Matthew Meyerson; of U.S.
Provisional Application No. 60/054,549, filed August 1,
1997, entitled "hEST2, the Putative Human Telomerase
Catalytic Subunit Gene Is Up-Regulated in Tumor Cells and
20 During Immortalization" by Matthew Meyerson, Christopher M.
Counter and Robert A. Weinberg; of U.S. Provisional

-2-

Application No. 60/055,762, filed August 14, 1997, entitled "Human Telomerase Catalytic Subunit Gene and Uses Therefor" by Matthew Meyerson, Christopher M. Counter and Robert A. Weinberg; and of U.S. Provisional Application No.

5   60/064,322, filed October 30, 1997, entitled "Human Telomerase Catalytic Subunit Gene and Uses Therefor", by Matthew Meyerson, Christopher M. Counter and Robert A. Weinberg. The entire teachings of these five referenced applications are expressly incorporated herein by

10   reference.

BACKGROUND OF THE INVENTION

In most eukaryotes, the telomere ends of linear chromosomes are replicated by the ribonucleoprotein enzyme telomerase (Blackburn, E.H. *Annu. Rev. Biochem. 53*, 163-94

15   (1984); Zakian, V.A., *Science 270*, 1601-7 (1995); Greider, C.W. & Blackburn, E.H., *Cell 43*, 405-13 (1985)). The RNA subunit of this enzyme has now been described in multiple species (Blasco, M.A. et al., *Science 269*, 1267-70 (1995); Feng, J., et al., *Science 269*, 1236-41 (1995); Greider,

20   C.W. & Blackburn, E.H., *Nature 337*, 331-7 (1989); Lingner, J. et al., *Genes Dev. 8*, 1984-98 (1994); McEachern, M.J. & Blackburn, E.H., *Nature 376*, 403-9 (1995); Melek, M. et al., *Mol. Cell Biol. 14*, 7827-38 (1994); Shippen-Lentz, D. & Blackburn, E.H., *Science 247*, 546-52 (1990); Singer, M.S.

25   & Gottschling, D.E., *Science 266*, 404-9 (1994)). Candidate protein subunits have been isolated in the ciliate *Tetrahymena* (Collins, K. et al., *Cell 81*, 677-86 (1995) but not, however, in any genetically tractable organism.

Telomerase is repressed in normal human somatic cells

30   but is re-activated during tumor progression. This re-

activation is not reflected by changes in the levels of
previously cloned genes encoding telomerase subunits.

SUMMARY OF THE INVENTION

Described herein are genes which are required for
5   telomerase enzymatic activity and the respective encoded
messenger RNAs (transcripts) and proteins. As described
below, the present invention relates to DNA which is
involved in telomere length regulation in eukaryotes and
proteins which are physically associated with the
10 respective active telomerase enzyme and, thus, are each a
component of the respective telomerase holoenzyme. In one
embodiment, the gene, RNA transcript and encoded protein
are a yeast gene and its encoded RNA transcript and
protein. In a second embodiment, the gene, RNA transcript
15 and encoded protein are human. Also described herein is a
human cDNA which encodes the human telomerase catalytic
protein subunit.

The yeast telomerase gene is present on chromosome XII
and its disruption alters telomere maintenance, as
20 demonstrated by the decrease in telomere length in
transposon-insertion mutants. The sequence of the gene,
referred to herein as *EST2* (SEQ ID NO.: 1), the deduced
amino acid sequence of the encoded *Est2* protein, also
referred to as Est2p, (SEQ ID NO.: 2) and the hEST2 RNA
25 transcript (SEQ ID NO.: 36) are provided. The DNA has been
shown to be essential for telomerase activity in yeast and
the encoded protein has been shown, by the methods
described herein, to be physically associated with
telomerase and a constituent of active telomerase complex
30 in yeast. The yeast telomerase DNA *EST2* is a subject of

-4-

the present invention; this includes isolated DNA
comprising DNA selected from the following: DNA of SEQ ID
NO.: 1; DNA which is the complement of SEQ ID NO.: 1, DNA
which hybridizes to DNA of SEQ ID NO.: 1 or a complement

5   thereof; DNA which localizes to yeast chromosome XII; and
DNA which encodes the amino acid sequence of SEQ ID NO.: 2.
Isolated yeast Est2p is also the subject of this invention.
This includes isolated Est2p of SEQ ID NO.: 2 and Est 2p
encoded by EST2 DNA as defined herein.

10   The gene described herein is useful to identify genes
encoding telomerase proteins in other eukaryotes,
particularly in vertebrates, including mammals and
especially humans. All or a portion of the gene described
can be used. The encoded portion can be used to produce

15   antibodies (monoclonal or polyclonal) which bind the *Est2*
protein and can, in turn, be used to identify corresponding
proteins (proteins physically associated with telomerase
enzymatic activity) in other eukaryotes.

Also described herein is a human cDNA, which was

20   originally named *hEST2* and has been renamed *hTERT*, that
encodes the human telomerase catalytic protein subunit, the
encoded *hEST2* RNA and the encoded hEST2 protein. The human
DNA described herein is a human homologue of yeast and
ciliate genes which encode telomerase catalytic subunits;

25   it shares significant sequence similarity with the
telomerase catalytic subunit genes, yeast *EST2* and Euplotes
p123. *hEST2* RNA expression reflects telomerase activity.
The RNA transcript is expressed in primary human tumors,
cancer cell lines and telomerase-positive tissues, but is

30   undetectable in telomerase-negative cell lines and
differentiated telomerase-negative tissues. *hEST2* message
is absent in pre-crisis, telomerase-negative transformed
cells, but is readily detectable in post-crisis,

telomerase-positive immortalized cells. Taken together, these observations are evidence that the induction of *hEST2* mRNA expression is required for the telomerase activation that occurs during cellular immortalization and tumor

5   progression.

The encoded human protein comprises six of the seven conserved sequence motifs which define the polymerase domains of members of the reverse transcriptase family and also includes the invariant aspartic acid residues required

10 for telomerase enzymatic activity. Although hEST2 protein comprises such motifs which define polymerase domains, beyond these it shows no sequence similarity with reverse transcriptases. It is more closely related to the telomerase catalytic subunits of yeast and ciliates than to

15 other reverse transcriptases. In its domains that lie N-terminal to the polymerase domain, hEST2 shows clear relatedness to both p123 and Est2p. Many of the sequence identities in the N-termini of the three proteins are in a region just before motif 1. These sequences do not appear

20 in reverse transcriptases or in other proteins and, thus, appear to be unique to telomerases. For example, these three include a unique motif, referred to as the telomerase motif; in hEST2 this motif extends from amino acid residue 556 to amino acid residue 565 of SEQ ID NO.: 3, with

25 absolute invariant sequence extending from amino acid residue 560 to amino acid residue 565 of SEQ ID NO.: 3. Further, within the hEST2 domain that shares sequence similarity with reverse transcriptases, it is clear that hEST2 is more closely related to telomerase reverse

30 transcriptases than to non-telomerase reverse transcriptases. The three telomerase catalytic subunits (hEST2, yeast EST2 and Euplotes p123) form a subgroup within the reverse transcriptase family.

The present invention relates to isolated DNA which encodes an RNA transcript which is expressed in primary human tumors, cancer cell line and telomerase-positive tissue. In one embodiment, the isolated DNA comprises

5 *hEST2* DNA such as DNA comprising SEQ ID NO.: 35 (DNA comprising the nucleotide sequence of SEQ ID NO.: 35). Isolated DNA of the present invention encodes an RNA transcript which is not detectable in telomerase-negative cell lines or in differentiated telomerase-negative

10 tissues. Further, isolated DNA of the present invention encodes an RNA transcript which is not detectable in pre-crisis, telomerase-negative transformed cells and is detectable in post-crisis, telomerase-positive immortalized cells. In a particular embodiment, the DNA comprises *hEST2*

15 cDNA, such as SEQ ID NO.: 35 or DNA which hybridizes thereto or to a complement of *hEST2* cDNA (such as a complement of SEQ ID NO.: 35) under conditions of high stringency. A further subject of the present invention is an isolated RNA transcript encoded by isolated DNA of the

20 present invention.

A method of altering telomerase function and, thus, of altering telomere shortening is also described. In the method, expression and/or function of the telomerase-associated protein is altered (enhanced or reduced),

25 resulting in altered (enhanced or reduced) cell lifespan. Expression of the telomerase-associated protein is enhanced, for example, by introducing DNA encoding the protein into cells. Alternatively, telomerase-associated protein is introduced into cells. Expression of the

30 telomerase-associated protein is reduced, for example, by introducing into cells an agent which inhibits production or function of the protein, an agent which destroys the expressed protein or a dominant negative form of the

protein. Therefore, the present invention also provides a
method of increasing the lifespan of a cell or,
alternatively, of reducing the lifespan of a cell, such as
that of cancer cells or transformed cells. In the method

5   of increasing lifespan of cells, telomerase activity is
maintained or increased within the cells, such that
telomeres are maintained and as the cells age, telomere
shortening does not occur or occurs to a lesser extent than
would otherwise be the case (if telomerase activity were

10 not maintained or increased). Reduction of lifespan of
cells, such as tumor cells, is accomplished by introducing
into the cells an inhibitor of the telomerase protein
subunit described herein.

            A method of assessing cells or aiding in the

15 assessment of cells for malignancy or an increased
likelihood of progression to malignancy is also the subject
of this invention. In the method, cells to be assessed are
obtained from an individual (e.g., a human) in need of such
an assessment. The cells are processed in such a manner

20 that DNA, RNA or both in the cells are rendered available
for annealing or hybridization with complementary
polynucleotides or oligonucleotides (DNA or RNA), such as
probes or primers, thereby producing processed cells. The
processed cells are combined with DNA or RNA required for

25 telomerase enzymatic activity (DNA or RNA encoding a
protein required for telomerase enzymatic activity), a
complement of the required DNA or RNA or a characteristic
portion or fragment of the DNA or RNA, such as the
telomerase motif or all or a portion of the 5' end of hEST2

30 which is not shared with the yeast or Euplotes gene. If
hybridization occurs, it is indicative of the presence of
telomerase protein-encoding DNA or RNA in the cells and of
activation of telomerase, which is also indicative of
malignancy or an increased likelihood of progression to

●

●

-8-

malignancy. As described herein, the *hEST2* DNA encodes an

RNA transcript which is expressed in primary tumors, cancer

cell lines and telomerase - positive tissues and is readily

detectable in post-crisis, telomerase-positive immortalized

5   cells, but not detectable in telomerase-negative cell lines

and differentiated telomerase-negative tissues. Detection

of *hEST2* DNA and/or of the RNA transcript, thus, makes it

possible to detect DNA which encodes a transcript required

for telomerase activation that occurs during cellular

10 immortalization and tumor progression and to detect the RNA

transcript itself.

In an alternative embodiment, the invention is a

method of diagnosing or aiding in the diagnosis of

development of malignancy (cancer, tumor formation) in an

15 individual, in which the occurrence (presence or absence)

and/or quantity of a telomerase protein (e.g., hEST2

protein) in cells is assessed. In the method, cells are

obtained from an individual in need of diagnosis of

malignancy and processed, if necessary, in such a manner

20 that proteins in the cell are available for detection, such

as by binding with antibodies or antibody fragments. The

processed cells are combined or contacted with antibodies

that recognize (bind) the telomerase protein (e.g.,

antibodies that bind Est2p or hEST2 protein) under

25 conditions appropriate for antibody binding to occur.

Whether binding occurs is determined; optionally, the

extent to which binding occurs can also be determined. If

binding of anti-telomerase protein antibodies (e.g., anti-

hEST2 protein antibodies) to a component of processed cells

30 occurs, it is indicative of the presence of hEST2 protein

in the cells and of malignancy or an increased likelihood

of development or malignancy in the individual.

In one embodiment, the present invention is a method

of reducing expression of *hEST2* RNA and hEST2 protein in

cells of an individual (e.g., a human or other mammal).
The method comprises administering to the individual a drug
selected from the group consisting of drugs which inhibit
(directly or indirectly) or bind *hEST2* RNA and prevent or

5   reduce production of hEST2 protein and drugs which inhibit
hEST2 protein function or activity. The drug is
administered in a therapeutically effective amount (an
amount sufficient to have the desired effect of reducing
expression of *hEST2* RNA and hEST2 protein) and under

10   conditions appropriate for entry into cells, in which they
have the desired effect.

In a further embodiment, the present invention is a
method of treating cancer in an individual, in need of such
treatment. In the method, a drug which inhibits or binds

15   *hEST2* RNA (or DNA) and prevents or reduces production of
hEST2 protein or a drug which inhibits hEST2 protein
function or activity is administered to the individual
under conditions appropriate for entry into cells and in a
therapeutically effective amount (an amount sufficient to

20   have the desired effect of inhibiting *hEST2* RNA and/or
preventing or reducing production of hEST2 protein or
inhibiting hEST2 protein function or activity), with the
result that cancer in the individual is treated (reduced,
reversed or prevented from advancing).

25   A further embodiment of the invention is a method of
altering (increasing or reducing) lifespan of cells in
culture or in an individual. In the method in which
lifespan is increased, *hEST2* DNA is introduced into cells
(e.g., in culture or in the individual), in which the *hEST2*

30   RNA transcript and encoded protein are produced in
sufficient quantity to increase the lifespan of cells in
the cultured cells or the individual. In the method in
which lifespan is decreased, hEST2 protein function,

activity or production is reduced (partially or totally).
This is done, for example, by introducing into cells in
culture or administering to an individual in whom cell
lifespan is to be decreased a drug which inhibits or binds
5   *hEST2* RNA and prevents or reduces production of hEST2
protein; a drug which inhibits hEST2 protein function,
activity or production; or a drug which inhibits or binds
hEST2 DNA and prevents or reduces production of *hEST2* RNA
transcripts. The drug is administered to an individual
10 under conditions appropriate for entry into cells in
sufficient quantity to have the desired effect (in
sufficient quantity to decrease lifespan of the cells by
reducing hEST2 protein function, activity or production).

In the above-described embodiments, the drug can be,
15 for example, a small organic molecule, an enzyme which
degrades hEST2 protein, an enzyme inhibitor (e.g., a
telomerase or hEST2 enzyme inhibitor), an hEST2
transcriptional regulator; an antisense molecule or a
dominant negative form of hEST2 protein.
20   Also described herein is a mutant yeast strain,
referred to as DN, which carries an inactive chromosomal
rad52Δ allele. The RAD 52 coding sequence of a haploid
strain of genotype *MATa leu 2-3, 112 lys2-201 trp1-1 ura3-
52 his3-200* was replaced with a selectable marker (e.g.,
25 the *HIS3* gene), to generate strain DN. Mutant yeast strain
DNR, in which the absence of *RAD52* was complemented by
introducing the plasmid *pYPCR+*[35], which encodes the *RAD52*
gene under it own promoter and the selectable marker *URA3*,
is also described. Also described are mutant yeast strain
30 *DNRtlcΔ*, in which the TLC1 gene in DNR cells was replaced
with the LEU2 selectable marker gene and Y0035, in which

the Est2 gene has been mutated with the addition of a TN3
LEU2 transposon in the DNR genetic background.

     The mutant yeast strain described herein requires
telomerase activity for viability. It is useful to carry
5   out a rapid and automatable biological assay for telomerase
inhibitors and, thus, for inhibitors of telomere
biosynthesis. The assay is also the subject of the present
invention. The mutant yeast strain is also useful to
identify agents which are recombination inhibitors. In the
10 mutant yeast strain, referred to as DN, the *RAD52*
recombination gene has been replaced by a *HIS3* gene. As
described herein, disruption of the *TLC1* telomerase RNA
subunit gene or the *MIT1* putative telomerase protein
subunit gene in the *rad52* mutants leads to a delayed cell
15 death. Yeast lacking both recombination activity and
telomerase activity die after about 20 to 60 cell
generations. Telomerase activity can be disrupted by
enzyme inhibitors, as well as by genetic inactivation.
Thus, for a telomerase inhibitor screen, test compounds are
20 added, at different concentrations, to the DNA mutant
strain (the test strain) and to a wild-type (control)
strain. Periodically, such as each day, cultures of the
two strains are diluted (e.g., 1:1000). Compounds that
kill the yeast instantly or that kill the DNA and wild-type
25 yeast with identical delays, are eliminated. Compounds
that kill the test yeast strain significantly before the
control yeast strain are telomerase inhibitors. This
biological assay has several advantages over available
methods. It is fast, automatable, and can be performed
30 easily in large quantities. Because it is a biological
assay, it reflects the physiological function of the drugs.
Furthermore, it allows testing of biochemical and uptake
parameters in a single step. Finally, this assay also

-12-

excludes inhibitors of other metabolic enzymes, such as RNA
and DNA polymerases and is specific for telomerase
inhibitors.

BRIEF DESCRIPTION OF THE DRAWINGS

5   Figures 1A and 1B present the nucleic acid sequence of
the *EST2* gene (SEQ ID NO.: 1) and the predicted amino acid
sequence of the Est2 protein (SEQ ID NO. 2).

Figure 2 shows alignment of the predicted amino acid
sequence of hEST2, also referred to as *hTERT*, (SEQ ID NO.:

10   3) with the yeast Est2p (SEQ ID NO.: 2) and Euplotes p123
(SEQ ID NO.: 4) homologues. Amino residues within shaded
blocks are identical between at least two proteins.
Identical amino acids within the reverse transcriptase (RT)
motifs (Poch *et al.*, *EMBO J.* *8*:3867-3874 (1989); Xiong and

15   Eickbush, *EMBO J.* *9*:3353-3362 (1990)) are in black boxes,
an example of a telomerase-specific motif in an outlined
gray box, and all other identical amino acids in gray
boxes. RT motifs are extended in some cases to include
other adjacent invariant or conserved amino acids. The

20   sequence of the expressed sequence tag AA281296 is
underlined.

Figures 3A-3F show the alignment of RT motifs 1 to 6
of telomerase subunits hEST2 (SEQ ID NO.: 5-10), p123 (SEQ
ID NO.: 11-16) and Est2p (SEQ ID NO.: 17-22) with S.

25   cerevisiae group II intron-encoded RTs a2-Sc (SEQ ID NO.:
23-28) and a1-Sc (SEQ ID NO.: 29-34). The consensus
sequence of each RT motif is shown (h: hydrophobic
residues, p: small polar residues, c: charged residues).
Amino acids that are invariant among telomerases and the RT

30   consensus are boxed in black, while those that are
invariant among telomerases and similar to the RT consensus
are boxed in gray. Open boxes identify highly conserved

-13-

residues unique to either telomerases or to non-telomerase RTs. Asterisks denote amino acids essential for polymerase catalytic function.

     Figure 4 is an ideogram of human chromosome 5p showing

5   linkage of *hEST2* to sequence-tagged site (STS) markers WI-9907 and D5S417 determined by radiation hybrid (RH) mapping employing the Genebridge 4 RH panel (GB) (1 cR=270 kb). The higher resolution Stanford G3 RH panel (G3) placed *hEST2* close to marker D5S678 (1 cR=30 kb). The

10   calculated distance between *hEST2* and these STS markers is in centiRays (cR) with LOD scores in parentheses.

     Figures 5A-5B show the nucleotide sequence of *hEST2* cDNA in which the start codon ATG is underlined (SEQ ID NO.: 35).

15   Figure 6 is the amino acid sequence of hEST2 (SEQ ID NO.: 3).

     Figures 7A, 7B and 7C are the nucleotide sequence of partially or alternatively spliced hEST2 message RNA (SEQ ID NO.: 36).

20   Figures 8A-8F show the expression of hEST2 in normal human tissues and cancer cell lines. Duplicate RNA blots were probed with an hEST2 probe (top panels) or with a β-actin probe as internal control (bottom panels). The 4.4 kb, 6 kb, and 9.5 kb transcripts are hEST2-specific.

25   Figures 9A-9D shows hEST2 expression in primary human tumors. Rnase protection assays are shown for hEST2 and β-actin controls. Sizes of the full-length and protected bands are indicated. Shown are the HL60 leukemia cell line (control), normal breast tissue, 2 primary breast tumors,

30   the MCF7 and T47D breast cancer cell lines, and normal and primary tumor tissues from the testis, colon, and ovary. The doublet seen protected by the hEST2 probe is invariant, and may be a result of probe secondary structure.

-14-

DETAILED DESCRIPTION OF THE INVENTION

The linear chromosomes of eukaryotic cells offer the biological advantages of rapid recombination, assortment, and genetic diversification. However, linear DNA is
5   inherently more unstable than circular forms. To address this difficulty, the eukaryotic chromosome has evolved to include a DNA-protein structure, the telomere, which caps chromosome ends and protects them from degradation and end-to-end fusion (Blackburn, *Ann. Rev. Biochem.* *53*:163-194
10   (1984); Zakian, *Science* *270*:1601-1607 (1995)).

The DNA component of telomeres consists of tandem repeats of guanine-rich sequences that are essential for telomere function (Blackburn, *Ann. Rev. Biochem.* *53*:163-194 (1984), Blackburn, *Nature* *350:569-573* (1991); Zakian,
15   *Science* *270*:1601-1607 (1995)). These repeats are replicated by conventional DNA polymerases and by a specialized enzyme, telomerase (Greider, In *Telomeres*, eds. Blackburn and Greider, Cold Spring Harbor Laboratory Press, pp. 35-68 (1995)), first identified in the ciliate
20   Tetrahymena (Greider and Blackburn, *Cell* *43*:405-413 (1985)). The telomerase enzyme is essential for complete replication of telomeric DNA because the cellular DNA-dependent DNA polymerases are unable to replicate the ultimate ends of the telomeres due to their requirement for
25   a 5' RNA primer and their unidirectional mode of synthesis. Removal of the most terminal RNA primer following priming of DNA synthesis leaves a gap that cannot be replicated by these polymerases (Olovnikov, *Dokl. Akad. Nauk SSSR* *201*:1496-1499 (1971); Watson, *Nature New Biol.* *239*:197-201
30   (1972)). Telomerase surmounts this problem by *de novo* addition of single-stranded telomeric DNA to the ends of chromosomes (Greider and Blackburn, *Cell* *43*:405-413 (1985),

Greider and Blackburn, *Nature 337*:331-337 (1989); Yu *et al.*, *Nature 344*:126-132 (1990); Greider, In *Telomeres*, eds. Blackburn and Greider, Cold Spring Harbor Laboratory Press, pp. 35-68 (1995)).

5   The telomerase enzymes that have been characterized to date are RNA-dependent DNA polymerases which synthesize the telomeric DNA repeats using an RNA template that exists as a subunit of the telomerase holoenzyme (Greider, In *Telomeres*, eds. Blackburn and Greider, Cold Spring Harbor

10   Laboratory Press, pp. 35-68 (1995)). The genes specifying the RNA subunits of telomerases have been cloned from a wide variety of species, including humans (Feng *et al.*, *Science 269*:1236-1241 (1995); Greider, In *Telomeres*, eds. Blackburn and Greider, Cold Spring Harbor Laboratory Press,

15   pp. 35-68 (1995)) and been shown in several instances to be essential for telomerase function *in vivo* (Yu *et al.*, *Nature 344*:126-132 (1990); Yu and Blackburn, *Cell 67*:823-832 (1991); Singer and Gottschling, *Science 266*:404-409 (1994); Cohn and Blackburn, *Science 269*:396-400 (1995);

20   McEachern and Blackburn, *Nature 376*:403-409 (1995)). In addition, three proteins have been identified to date that are associated with telomerase activity. p80 and p95 were purified from the ciliate Tetrahymena (Collins *et al.*, *Cell 81*:677-686 (1995)) and the gene encoding a mammalian

25   homologue of p80, TP1/TLP1, has also been cloned (Harrington *et al.*, *Science 275*:973-977 (1997); Nakayama *et al.*, *Cell 88*:875-884 (1997)). The specific mechanism by which these proteins participate in telomerase function has not been defined.

30   Perturbing the function of telomerase in the ciliate Tetrahymena, through the overexpression of an inactive form

of the telomerase RNA, or in yeast, through the mutation of
genes encoding either the catalytic protein or template RNA
subunit, leads to progressive telomere shortening as cells
pass through successive cycles of replication (Yu *et al.*,

5   *Nature 344*:126-132 (1990); Singer and Gottschling, *Science
266:404-409 (1994); McEachern and Blackburn, *Nature
376:403-409 (1995); Lendvay *et al.*, *Genetics 144*:1399-1412
(1996); Counter *et al.*, *Proc. Natl. Acad. Sci.*, *USA
94:9202-9207 (1997); Lingner *et al.*, *Science 276*:561-567

10   (1997)). This loss of telomeric DNA is ultimately lethal
if it is not overcome. The lethality seems to be triggered
when telomeres have been truncated below a critical
threshold level. Hence, in the absence of compensating
mechanisms, yeast cell lineages that lack telomerase

15   activity have a lifespan dictated by the lengths of their
telomeres.

      In humans, telomerase activity is readily detectable
in germline cells and in certain stem cell compartments.
However, enzyme activity is not detectable in most somatic

20   cell lineages (Harley *et al.*, *Cold Spring Harb. Symp.
Quant. Biol. 59*:307-315 (1994); Kim *et al.*, *Science
266:2011-2015 (1994); Broccoli *et al.*, *Proc. Natl. Acad.
Sci.*, *USA 92*:9082-9086 (1995); Counter *et al.*, *Blood
85:2315-2320 (1995); Hiyama *et al.*, *J. Immunol. 155*:3711-

25   3715 (1995)). Consistent with this, telomeres of most
types of human somatic cells shorten with increasing
organismic age and with repeated passaging in culture,
similar to the situation seen in protozoan and yeast cells
that have been deprived experimentally of a functional

30   telomerase enzyme (Harley *et al.*, *Nature 345*:454-460
(1990); Hastie *et al.*, *Nature 346*:866-868 (1990)).

-17-

Eventually the proliferation of cultured human cells will
halt at a point termed senescence (Hayflick and Moorhead,
Exp. Cell. Res. *25*:585-621 (1961); Goldstein, *Science*
249:1129-1133 (1990)), apparently before the telomeres of
5   these cells have become critically short.

Cultured normal human cells can circumvent senescence
and thereby continue to proliferate when transformed by a
variety of agents. In such cultures, telomere shortening
continues until a subsequent point is reached that is
10   termed crisis, where telomeres have become extremely short
(Counter *et al.*, *EMBO J.* *11*:1921-1929 (1992) and *J. Virol.*
68:3410-3414 (1994); Shay *et al.*, *Oncogene* *8*:1407-1413
(1993); Klingelhutz *et al.*, *Mol. Cell. Biol.* *14*:961-969
(1994). Crisis, perhaps best described in SV-40
15   transformed cells, is characterized by karyotypic
instability, particularly the types of instability observed
in chromosomes lacking functional telomeres, and by
significant levels of cell death (Sack, *In Vitro* *17*:1-19
(1981)). The crisis phenotype is reminiscent of that
20   observed in yeast and Tetrahymena cells in which telomerase
function has been experimentally perturbed.

The simplest interpretation of these data is that the
lifespan of telomerase-negative human cells, like that of
their yeast and ciliate counterparts, is ultimately limited
25   by the length of telomeres. Rare human cells that have
acquired the ability to grow indefinitely emerge from
crisis populations with a frequency of 10^{-6} to 10^{-7}
(Huschtscha and Holliday, *J. Cell Sci.* *63*:77-99 (1983);
Shay and Wright, *Exp. Cell Res.* *184*:109-118 (1989)). This
30   implies that a mutational event is required to confer the
immortal phenotype on these cells. The immortal cells that
escape crisis are characterized by readily detectable

●   ●

-18-

levels of telomerase activity and by stable telomeres
(Counter et al., EMBO J. 11:1921-1929 (1992) and J. Virol.
68:3410-3414 (1994); Shay et al., Mol. Cell. Biol. 15:425-
432 (1995); Whitaker et al., Oncogene 11:971-976 (1995);

5   Gollahon and Shay, Oncogene 12:715-725 (1996); Klingelhutz
et al., Nature 380:79-82 (1996)). This suggests that
activation of telomerase can overcome the limitations
imposed by telomere length on the lifespan of cell
lineages.

10   Activation of telomerase also appears to be a major
step in the progression of human cancers. Unlike normal
human cells, cancer cells can be established as permanent
cell lines and, thus, are presumed to have undergone
immortalization during the process of tumorigenesis.

15   Moreover, telomerase activity is readily detected in the
great majority of human tumor samples analyzed to date
(Counter et al., Proc. Natl. Acad. Sci., USA 91:2900-2904
(1994); Kim et al., Science, 266: 2011-2015 (1994); Shay
and Bacchetti, Eur. J. Cancer 33:787-791 (1997)).

20   Taken together, these various observations have been
incorporated into a model which proposes that the
limitation on prolonged cell replication imposed by
telomere shortening serves as an important anti-neoplastic
mechanism used by the body to block the expansion of

25   pre-cancerous cell clones. According to such a model,
tumor cells transcend the crisis barrier and emerge as
immortalized cell populations by activating previously
unexpressed telomerase, enabling them to restore and
maintain the integrity of their telomeres (Counter et al.,

30   EMBO J. 11:1921-1929 (1992) and Proc. Natl. Acad. Sci., USA
91:2900-2904 (1994); Harley et al., Cold Spring Harb. Symp.
Quant. Biol. 59:307-315 (1994)).

-19-

A major question provoked by this model is the
mechanism used to resurrect telomerase expression during
tumor progression. Expression of the telomerase-associated
protein TP1/TLP1 does not reflect the level of telomerase

5 activity (Harrington *et al.*, *Science 275*:973-977 (1997);
Nakayama *et al.*, *Cell 88*:875-884 (1997)). It is also clear
that the levels of the human telomerase RNA component, *hTR*,
cannot completely explain the regulation of telomerase
activity. Although the levels of *hTR* and its mouse

10 counterpart, *mTR*, increase with tumor progression (Feng *et*
al., *Science 269*:1236-1241 (1995); Blasco *et al.*, *Nat.*
Genet. 12:200-204 (1996); Broccoli *et al.*, *Mol. Cell. Biol.*
16:3765-3772 (1996); Soder *et al.*, *Oncogene 14*:1013-1021
(1997)), the amounts of these transcripts do not always

15 correlate with enzymatic activity. Indeed, *hTR* or *mTR*
transcript levels can be significantly higher in
telomerase-negative cells and tissues than in
telomerase-positive cancer cells (Avilion *et al.*, *Cancer*
Res. 56:645-650 (1996); Bestilny *et al.*, *Cancer Res.*

20 *56*:3796-3802 (1996); Blasco *et al.*, *Nat. Genet. 12*:200-204
(1996)). Similarly, even though telomerase levels increase
100- to 2000-fold during the immortalization of human
cells, the level of *hTR* message increases at most two-fold
(Avilion *et al.*, *Cancer Res. 56*:645-650 (1996). Therefore,

25 derepression of the *hTR* and TP1 subunits cannot easily be
invoked to explain the appearance of telomerase activity in
the great majority of human tumor samples. Thus far, the
rate-limiting step in telomerase activation has remained
elusive.

30 Described herein is work which has shown that
mutations in a gene of eukaryotic origin (in an open

reading frame required for telomere maintenance) results in
loss (absence) of telomerase enzymatic activity in cells
and that the encoded protein is physically associated with
the active telomerase enzyme, thus showing that the DNA
5   encodes a component of a eukaryotic telomerase holoenzyme.
Also described herein is isolated DNA of eukaryotic origin
which is required for telomerase enzymatic activity and the
isolated encoded RNA transcripts and proteins.
Specifically, EST2, a yeast gene, and hEST2, which is of
10   human origin and the encoded respective transcripts and
proteins (yeast EST2p and human hEST2) are described, as
are their respective sequences. DNA of the present
invention includes cDNA, genomic DNA, recombinantly
produced DNA and chemically synthesized DNA. It can be
15   obtained from a source in which it occurs in nature or can
be produced using known chemical or recombinant DNA
methods. The following is a discussion of work carried out
relating to yeast and human telomerase protein.

Gene Encoding Protein Subunit Of Yeast Telomerase
20   Holoenzyme and the Encoded Protein
     As described herein, it has been shown, using a
genetic screen in budding yeast, that mutations in EST2, an
open reading frame required for telomere maintenance,
(Lendvay, T.S. et al., Genetics 144, 1399-1412 (1996)
25   result in the absence of telomerase enzymatic activity in
cells. As also described herein, epitope tagging and
immunochemical studies showed that the Est2 protein is
physically associated with the active telomerase enzyme.
Thus, EST2 encodes a component of the yeast telomerase
30   holoenzyme.
     Interest in telomeres and telomerase has heightened in
recent years with the discovery that telomerase is present

-21-

at low, almost undetectable, levels in most human somatic tissues and is readily detectable in germline cells and in the vast majority of tumor cell samples (Counter, C.M. *et al.*, *Proc. Natl. Acad. Sci. USA 91*, 2900-4 (1994); Kim,

5   N.W. *et al.*, *Science 266*, 2011-5 (1994). Somatic cell lineages which lack telomerase lose telomere segments progressively as they pass through successive replication cycles, limiting their lifespan (Harley, C.B. *et al.*, *Nature 345*, 458-60 (1990), Hastie, N.D. *et al.*, *Nature 346*,

10   866-8 (1990)). Conversely, cell populations that resurrect telomerase expression ensure maintenance of telomere length, thereby removing a barrier to their further unlimited replication and resulting in the process termed immortalization (Counter, C.M. *et al.*, *Embo. J. 11*, 1921-9

15   (1992)). These observations have led to a model which proposes that the process of telomere shortening limits the replicative potential of most human somatic cell lineages, and that cancer cells overcome this limitation by activating telomerase expression during the course of tumor

20   progression (Counter, C.M. *et al.*, *Embo. J. 11*, 1921-9 (1992); Harley, C.B. *et al.*, *Cold Spring Harb. Symp. Quant. Biol. 59*, 307-15 (1994)).

Validation of this model has been held back by the difficulties associated with isolating and characterizing

25   the telomerase enzyme and its encoding genes. Much of this work has focused on the telomerase enzymes of ciliates; the formation of thousands of mini-chromosomes present in their macro-nuclei seems to require correspondingly high amounts of telomerase, dwarfing the amounts available for study in

30   metazoan cells. The telomerase enzyme in *Tetrahymena* has been reported to be composed of two proteinaceous and one RNA subunit, the latter responsible for templating the

-22-

telomeric DNA segments (Greider, C.W. & Blackburn, E.H.,
Nature 377, 331-7 (1989); Collins, K. *et al.*, *Cell 81*,
677-86 (1995); Yu, G.L. *et al.*, *Nature 344*, 126-32 (1990)).
The protein subunits of telomerase have not been described
5   in any other organism, although the genes specifying the
RNA subunits of telomerase have been cloned in a wide range
of other species (Blasco, M.A. *et al.*, *Science 269*, 1967-70
(1995); Feng, J., *et al.*, *Science 269*, 1236-41 (1995);
Lingner, J. *et al.* , *Genes Dev. 8*, 1984-98 (1994);
10   McEachern, M.J. & Blackburn, E.H., *Nature 376*, 403-9
(1995); Melek, M. *et al.*, *Mol. Cell Biol. 14*, 7827-38
(1994); Shippen-Lentz, D. & Blackburn, E.H., *Science 247*,
546-52 (1990); Singer, M.S. & Gottschling, D.E., *Science
266*, 404-9 (1994)).

15   Genetic strategies designed to identify and isolate
telomerase-encoding genes have centered on the yeast, *S.
cerevisiae*. Mutations in several genes have been found to
lead to progressive telomere shortening. Of these genes,
TLC1, which specifies the RNA subunit of the telomerase
20   enzyme (Singer, M.S. & Gottschling, D.E., *Science 266*, 404-
9 (1994)), is required for telomerase enzymatic activity
(Cohn, M. & Blackburn, E.H., *Science 269*, 396-400 (1995);
Lin, J.J. & Zakian, V.A., *Cell 81*, 1127-35 (1995); Lue,
N.F. & Wang, J.C., *J. Biol. Chem. 270*, 21453-6 (1995);
25   Steiner, B.R. *et al.*, *Proc. Natl. Acad. Sci. USA 93*, 3817-
21 (1996)). On the other hand, *EST2* encodes a protein of
unknown function (Lendvay, T.S. *et al.*, *Genetics 144*, 1399-
1412 (1996)); *EST1* (Lundblad, V. & Szostak, J.W., *Cell 57*,
633-43 (1989)) and *CDC13* (Garvik, B. *et al.*, *Mol. Cell
30   Biol. 15*, 6128-38 (1995); Nugent, C.I., Hughes, T.R., Lue,

N.F. & Lundblad, V., *Science 274*, 249-52 (1996)) are not
essential for telomerase activity (Cohn, M. & Blackburn,
E.H., *Science 269*, 396-400 (1995); Nugent, C.I. *et al.*,
Science 274, 249-52 (1996)); and *KEM1* appears to encode a

5 nuclease that acts on telomeres (Liu, Z. & Gilbert, W.,
Cell 77, 1083-92 (1994); Liu, Z. *et al.*, *Proc. Natl. Acad.
Sci. USA 92*, 6002-6 (1995)). A search for genes encoding
telomerase protein subunits was initiated because the
protein subunits of the yeast telomerase have not yet been

10 identified, and because there are no obvious homologs of
the *Tetrahymena* telomerase protein subunits encoded in the
yeast genome (Collins, K. *et al.*, *Cell 81*, 677-86 (1995);
Cherry, J.M. *et al.*, Saccharomyces Genome Database (http//
genome-www.stanford.edu/Saccharomyces).

15   A genetic screen designed to identify the genes
specifying telomerase subunits was undertaken, based on the
observation that telomere loss in yeast cells lacking the
telomerase RNA component can be compensated by the actions
of a second telomere-maintenance system that utilizes the

20 DNA recombination machinery. An essential component of
this machinery is the product of the *RAD52* gene.
Accordingly, the simultaneous inactivation of *RAD52* and the
telomerase RNA component results in cell lethality in both
S. cerevisiae and *K. lactis* (Lendvay, T.S. *et al.*, *Genetics*

25 *144*, 1399-1412 (1996); McEachern, M.J. & Blackburn, E.H.,
Genes Dev. 10, 1822-34 (1996)). This lethality is only
observed after some delay and occurs in concert with the
extensive telomere shortening observed following serial
passaging of the mutant cells. Applicants reasoned that

30 mutation of other genes contributing to telomerase
function, including the genes encoding the protein

-24-

subunit(s) of the enzyme, might be recognized because they
similarly render the yeast dependent on wild-type *RAD52* for
prolonged viability. If so, telomerase mutant yeast could
be identified by their requirement for *RAD52* function.

5   A yeast strain carrying an inactive chromosomal *rad52Δ*
allele that was complemented by a plasmid-borne wild-type
RAD52 gene linked to a *URA3* gene was generated, in order to
determine if mutants in telomerase function could be
revealed by a screen for mutants that require *RAD52*

10 function. When *RAD52* function was inactivated through
eviction of the *RAD52*-encoding plasmid (by counter-
selecting against the URA3 marker with 5-fluoro-orotic acid
(FOA), cell viability was unaffected for the succeeding 80
cell generations (Boeke, J.D. *et al.*, *Mol. Gen. Genet.* *197*,

15 345-6 (1984). However, when the *TLC1* gene, which encodes
the RNA subunit of telomerase, was additionally inactivated
in this strain, a decrease in cell viability was observed
after 60 generations. Moreover, when the rescuing *RAD52*
plasmid was evicted, there was a marginal decrease in

20 viability after 40 generations and complete inviability
after 60 generations. This showed that telomerase-
deficient cells perish upon the loss of *RAD52* function and
that this phenotype is therefore useful to screen for
mutants carrying lesions in a variety of genes affecting

25 telomerase function.

        The *rad52Δ* mutant cells carrying the complementing
RAD52 plasmid were mutagenized by homologous recombination
with a library of yeast genomic fragments, each of which
carried one or more copies of an inserted mini-

30 Tn3::*lacZ*::*LEU2* transposon (Burns, N., *et al.*, *Genes Dev.* *8*,
1087-105 (1994)). To ensure a high density of mutations,

1x10^6 transposon-mutagenized yeast clones were generated.
These mutagenized yeast cells were cultured for 40
generations to allow the telomeres of any cells lacking
telomerase function to shorten substantially. A portion of
5   the cell colonies was replica-plated and the *RAD52* plasmid
was evicted from one of each replica pair to identify cell
clones that required *RAD52* for continued viability.

Of the 10^5 transposon-mutagenized clones plated after
40 generations, approximately 2,500 reproducibly lost
10 viability following removal of the *RAD52* plasmid. In this
case, as earlier, these cells were placed in the presence
of FOA to select against the presence of the plasmid-borne
URA3 gene. The loss of viability following FOA might have
derived through a second, unrelated mechanism in which the
15 *URA3* gene became stabilized through chromosomal
integration, rendering cells FOA-sensitive independent of
any effects on telomeres. For this reason, the FOA-
sensitive cell clones were mated with wild-type yeast,
yielding diploids; all cell clones for which the diploid
20 derivatives remained FOA-sensitive were discarded.

The 245 remaining *RAD52*-dependent cell clones were
tested for changes in telomere structure by Southern
hybridization analysis of their telomeres. Of these 245
haploids, 16 cell clones carried short telomeres identical
25 to those seen in yeast lacking a functional *TLC1* gene.
Tetrad analysis of the diploid derivatives of these clones
was performed to determine whether the *rad52* synthetic
lethal phenotype was caused by the transposon insertion or
by an adventitious unlinked mutation elsewhere in the yeast
30 genome. In each case, the lethality co-segregated with the
transposon insertion, demonstrating that this phenotype is

derived directly from the genetic disruption effected by a single inserted transposon.

To determine which genes were disrupted by transposon insertion in the 16 yeast strains containing short
5 telomeres, the transposons together with flanking genomic DNAs from each strain were cloned and the DNA sequences flanking the transposon insertion site were analyzed. This sequencing revealed that 6 clones represented 6 distinct insertion sites within the *TLC1* gene which, as mentioned
10 above, specifies the RNA subunit of the telomerase. This result provided strong and direct validation of the utility of the strategy described to screen for genes that directly affect telomerase function.

Eight of the cell clones having shortened telomeres
15 and lethality in the absence of *RAD52* carried transposons that had inserted into seven distinct positions within an uncharacterized open reading frame on chromosome XII at nucleotides 766540 to 769194 (Cherry, J.M. *et al.*, Saccharomyces Genome Database (http//genome-www.stanford.
20 edu/Saccharomyces)).

The predicted amino acid sequence encoded by the gene identified on chromosome XII is presented in Figures 1A and 1B (SEQ ID NO.: 2); the predicted protein includes 884 amino acids with an estimated mass of 102 kilodaltons. The
25 predicted gene product does not share significant sequence similarity with any sequences in the databases available through the National Center for Biotechnology Information BLAST server. However, subsequent to completion of the work described herein, a report was published on the
30 identification of a novel gene, *EST2*, involved in telomere length regulation. The open reading frame identified on chromosome XII is identical to *EST2*; therefore, this gene is referred to herein as *EST2*.

Genetic analysis of the *est2* transposon-insertion
mutants shows that they have the same delayed cell death
phenotype, dependence on *RAD52*, and telomere shortening as
was displayed by cells carrying mutations in their *TLC1*

5   gene. Diploids heterozygous for disrupted alleles of *RAD52*
and *EST2* were sporulated and the resulting tetrads were
analyzed for growth in culture and for telomere length. In
33 tetrads analyzed, the vast majority of the *est2*::TN3
rad52Δ double mutant spore products became inviable by

10   approximately 20 generations in culture, and the remainder
died by approximately 40 generations. The viability of the
est2::Tn3 mutants with wild-type *RAD52* began to decline by
approximately 60 generations, and this phenotype was more
pronounced after approximately 80 generations. The *EST2*

15   wild-type spore products remained completely viable at all
generations, regardless of their *RAD52* genotype.
Consistent with the role of *EST2* in telomere maintenance,
the decrease in telomere length, by which this gene was
identified, co-segregated with the transposon insertional

20   mutations in *EST2*. This telomere shortening was comparable
to that observed in *tlcI*::TN3 mutants.

Until now, *TLC1* has been the only yeast gene known to
be a subunit of telomerase and to be required for telomere
activity *in vitro*. The *est2*::TN3 gene mutants are

25   phenotypically indistinguishable from *tlc1*::TN3 mutants.
Analyses were carried out to determine whether *EST2* is also
required for telomerase function.

A telomerase assay was used to show that extracts from
wild-type yeast catalyze the elongation of a single-

30   stranded telomeric primer by four to sixteen nucleotides in
an RNase-sensitive fashion, indicating telomerase activity.

However, extracts derived from yeast bearing transposons
inserted into EST2 lacked detectable telomerase activity,
like tlc1Δ mutants, which are known to be telomerase-
negative (Cohn, M. & Blackburn, E.H., Science 269, 396-400

5   (1995)). Comparison with dilutions of wild-type extract
made it possible to estimate that extracts from both tlc1
and est2 mutants contain at most 5% of wild-type telomerase
activity. While the actual levels of telomerase in the
mutant yeast are likely to be much lower, such lower levels

10 could not be quantified with this assay. As expected from
the phenotypic analysis of tetrads, the absence of
detectable telomerase activity co-segregated with
transposon insertion, independent of the RAD52 allele.

     The requirement of EST2 function for telomerase

15 activity suggests that Est2 is either a protein subunit of
telomerase or an upstream regulator required for telomerase
activity. It was possible to distinguish between these
possibilities by replacing the endogenous EST2 gene with a
variant encoding a protein with three influenza

20 hemagglutinin (HA) epitope stages at its carboxy terminus
(EST2-HA). This modified EST2 allele and, thus, its
product, is fully functional. The EST2-HA yeast show no
overt phenotype, their telomeres are wild-type length, and
extracts from these yeast exhibit normal levels of

25 telomerase activity. Extracts from EST2-HA and control
yeast were incubated with excess anti-HA antibody, after
which the immunoprecipitates and resultant supernatants
were separated and assayed for telomerase activity. The
anti-HA antibody immunoprecipitated telomerase activity

30 from extracts of yeast expressing the tagged Est2-HA
protein, but not from extracts expressing normal Est2
protein. The immunoprecipitation was specific for the

-29-

anti-HA antibody since a control anti-Myc epitope antibody failed to immunoprecipitate activity from either extract. Essentially all of the telomerase activity remained in the supernatant of the anti-HA immunoprecipitation from wild-

5 type extracts, whereas incubation with the anti-HA antibody almost completely immunodepleted the telomerase activity from the Est2-HA extracts. These data indicated that Est2 is not only physically associated with telomerase, but is a constituent of most, if not all, of the active telomerase

10 complex.

Intriguingly, *EST2* shares no homology with either of the two genes reported to encode the *Tetrahymena* telomerase protein subunits (Collins, K. *et al.*, *Cell 81*, 677-86 (1995)). A search of the yeast genome (Cherry, J.M. *et*

15 *al.*, Saccharomyces Genome Database (http//genome-www.stanford.edu/Saccharomyces) revealed no open reading frame sharing obvious sequence similarity with the *Tetrahymena* genes. Thus, the genes encoding the yeast and ciliate telomerase enzymes may be highly diverged, the Est2

20 function may be absent in *Tetrahymena*, or the analogous Est2 protein of *Tetrahymena* may not yet have been identified.

Work described herein supports the essential role of *EST2* gene for telomerase activity *in vitro* and *in vivo*.

25 Immunochemical experiments show that Est2-containing complexes contain telomerase activity, and that removal of these complexes from the cell concomitantly removes the telomerase activity. These characteristics argue that Est2 is an essential and integral component of the yeast

30 telomerase holoenzyme. The identification of this protein subunit of yeast telomerase should aid in the characterization of the other elements of this complex,

-30-

both in yeast and in other species, as well as in the understanding of the biochemical mechanism by which this enzyme acts.

5   Work described herein provides the basis for a method of assessing the ability of a molecule or compound to act as a telomerase inhibitor or as a recombination inhibitor. It provides a method by which a drug (molecule or compound) which inhibits telomerase or inhibits recombination can be identified. Eukaryotic linear chromosomes are capped with

10   telomeres, a DNA-protein complex that protects the integrity of the chromosome end. Telomeric DNA sequences are composed of multiple repeat elements. When the telomeric DNA is lost from chromosome ends, cells die because of the resulting chromosomal instability.

15   Telomere length is maintained by two mechanisms. In almost all eukaryotes growing under normal conditions, telomeric DNA repeats are synthesized by the ribonucleoprotein enzyme telomerase. This RNA-dependent DNA polymerase (or "reverse transcriptase") copies the DNA

20   repeats from an RNA template that is a component of the enzyme.

When the telomerase pathway is inactive, however, telomere length can be maintained by an alternative mechanism in yeast and possibly also in humans: the

25   homologous recombination pathway used for double strand break repair.

In the yeast *Saccharomyces cerevisiae*, mutants of the telomerase subunits in conjunction with mutants of the recombination pathway have been shown to become inviable

30   after growth for 40 to 80 generations. This synthetic lethal phenotype was used as a screen for telomerase mutants in a background of recombination mutants and, as a result, a novel telomerase gene was identified.

-31-

Drug screens which work in the same fashion as demonstrated herein for the genetic screen are useful to identify telomerase inhibitors in a recombination mutant background, and recombination inhibitors in a telomerase
5 mutant background.

Telomere maintenance inhibitors, which can be a telomerase inhibitor alone, a recombination inhibitor alone, or a combination, can be identified by the method described herein, using appropriate mutant eukaryotic
10 cells, such as the mutant yeast strain described herein. Such telomerase inhibitors can be further assessed, using known methods, to confirm their effectiveness as anti-fungal drugs or as anti-cancer drugs. Normal human somatic cells lack a pathway to maintain telomere lengths, and
15 their telomeres shorten continuously. Immortal human cells, whether in tissue culture or the vast majority of malignant tumors in the human body, have developed a mechanism to maintain telomeres. Telomere maintenance occurs as a result of activation of the telomerase enzyme,
20 activation of a recombination pathway, or possibly both. In yeast, the inactivation of both pathways has been shown to kill cells; in immortal tumor cells, pharmacological inhibitors must be tested before the effects of inactivation of either or both pathways can be assessed. A
25 brief description of screens for telomerase inhibitors and recombination inhibitors follows.

Screen for Telomerase Inhibitors

As described below, a mutant strain of the yeast *Saccharomyces cerevisiae* that requires telomerase activity
30 for viability has been generated. This strain can be used in a rapid and automatable biological assay for telomerase inhibitors.

-32-

In the mutant yeast strain, named DN, the *RAD52*
recombination gene has been replaced by a *HIS3* gene.
Disruption of the *TLC1* telomerase RNA subunit gene or the
MIT1/EST2 telomerase protein subunit gene in the *rad52*
5   mutants has been shown to lead to a delayed cell death.
Yeast lacking both recombination activity and telomerase
activity die after about 20 to 60 cell generations.

Telomerase activity can be disrupted by enzyme
inhibitors as well as by genetic inactivation, and,
10 therefore, the mutant strains described can be used to
identify telomerase inhibitors. For a telomerase inhibitor
screen, test compounds are added at different
concentrations to the DN mutant strain and to a wild-type
control strain. Each day, the cultures are be diluted
15 (e.g., 1:1000). Compounds that kill the yeast instantly,
or that kill the DN and wild-type yeast with identical
delays, are eliminated. Compounds that kill the DN yeast
significantly before the wild-type yeast are telomerase
inhibitors.

20   The method described is a biological assay and has
several advantages over available methods. It is fast,
automatable, and can be performed easily in large
quantities. As a biological assay, it reflects the
physiological function of the drugs. Furthermore, it
25 allows testing of biochemical and uptake parameters in a
single step. Finally, this assay will also exclude
inhibitors of other metabolic enzymes, such as RNA and DNA
polymerases and is highly specific for telomerase
inhibitors.

30   High-throughput screening can be accomplished by
growing the yeast in microtiter dishes. Aliquoting of
media, dilution of test drug compounds, and dilution of
yeast cultures can be performed robotically. Growth can be

-33-

measured by the optical density of the yeast culture at
595-600 nm; growth measurements and preliminary analysis of
the data can also be automated.

        The telomerase inhibitor screen has several
5   applications, such as to identify inhibitors of fungal
telomerase enzymes, mammalian telomerase enzymes and
reverse transcriptase.
        1)   Inhibitors of fungal telomerase enzymes. This screen
is a rapid method for identification of compounds that
10   inhibit telomerase in the yeast *Saccharomyces cerevisiae*.

This yeast is closely related to the fungal pathogen
Candida albicans and, therefore telomerase inhibitors

effective in *Saccharomyces* will likely function in *Candida*,

as well as in other pathogenic yeast. There are only a few
15   effective anti-fungal drugs currently available; many are
inadequate because of their serious side effects. Thus, a
novel approach to anti-fungal drugs could be clinically
valuable for the treatment of systemic fungal infections.
        2)   Inhibitors of mammalian telomerase enzymes. Telomerase
20   function is likely to be conserved between diverse species.
Therefore, it is highly possible that inhibitors of yeast
telomerase could also inhibit mammalian telomerase, and can
be tested for their abiltiy to act as anti-cancer agents.
These compounds can also serve as lead compounds for the
25   identification of such inhibitors and for modification to
produce more effective inhibitors (e.g., inhibitors which
are longer-lived, exhibit great inhibitory effects, resist
degradation by cellular enzymes).
        3)   Inhibitors of reverse transcriptase. The drugs that
30   have been identified as telomerase inhibitors to date have
been shown to be inhibitors of HIV reverse transcriptase.
Telomerase inhibitors identified by the present screen can

-34-

be assessed, using known methods, for their ability to act
as retroviral reverse transcriptase.

Screen for recombination inhibitors

     In this case, inhibitors of the telomere recombination
5   pathway are identified by their ability to kill telomerase
mutant yeast but not wild-type yeast. The screen is
similar to the telomerase inhibitor screen described above.
     For a recombination inhibitor screen, test compounds
are added at different concentrations to a telomerase
10   mutant strain (e.g., *tlc1* or *mit1/est2*), referred to as a
test strain, and to a wild-type control strain. Each day,
the cultures are diluted 1:1000. Compounds that kill the
yeast instantly, or that kill the telomerase mutant and
wild-type yeast with identical delays, are eliminated.
15   Compounds that kill the telomerase mutant yeast
significantly before the wild-type yeast are recombination
inhibitors. The ability of the compounds to inhibit
recombination can be confirmed using known methods. The
recombination inhibitors have several uses. For example,
20   combination therapy of a recombination inhibitor with a
telomerase for treating fungal infections and/or malignant
neoplasms. In addition, recombination inhibitors can be
used to potentiate the toxic effects of radiation treatment
and of chemotherapeutic agents that induce DNA double-
25   strand breaks.
     The following methods and materials were used in the
work described herein.

METHODS
     Yeast strains: Strain DN was generated by replacing
30   the *RAD52* coding sequence of the haploid strain L3853 (gift
of G. Fink; genotype *MATa leu2-3,112 lys2-201 trp1-1 ura3-*

52 his3-200) with the *HIS3* gene using a PCR-based
homologous gene disruption method (Baudin, A. *et al.,*
Nucleic Acids Res. 21, 3329-30 (1993)). The absence of
RAD52 in this strain was complemented by introducing the

5   plasmid pYPCR+[35], which encodes the *RAD52* gene under its
own promoter and the selectable marker *URA3*, thereby
generating strain DNR. The *TLC1* gene was replaced with the
LEU2 selectable marker by the above PCR procedure in L3853
and DNR cells, yielding the strains *tlc1Δ* and *DNRtlcΔ,*

10   respectively.
        Yeast mutagenesis: Approximately 230 µg of the
appropriately prepared yeast::mini-Tn3::*lacZ::LEU2* genomic
library (Burns, N., *et al., Genes Dev. 8,* 1087-105 (1994))
were introduced by homologous recombination into

15   approximately 7×10^6 cells of strain DNR. Approximately
1×10^6Ura$^+$Leu$^+$ clones representing successful transposon
insertions were pooled and replated to a total number of
~1×10^6 clones to "age" the yeast for the *rad52Δ* synthetic
lethal screen. 1×10^5 of such yeast were then reseeded for

20   the *rad52Δ* synthetic lethal screen, as described in the
text. DNA flanking insertion sites were rescued as
described by Burns, sequenced and compared to the
Saccharomyces Genome Database (Stanford University School
of Medicine, Department of Genetics) (Burns, N., *et al.,*

25   *Genes Dev. 8,* 1087-105 (1994)).
        Southern hybridization: Genomic DNA was isolated and
digested with the restriction enzyme *Xho*I, which liberates
the telomeric DNA (Guthrie, C., & Fink, G.R., *Guide to*
Yeast Genetics and Molecular Biology (Academic Press, new

30   York, 1991); Chan, C.S. & Tye, B.K., *Cell 33,* 563-73

-36-

(1983)). 2 μg of this DNA was resolved on 0.8% agarose gel for 570 Vh, and Southern hybridized as described by Counter *et al.* using a ^{32}P kinase labelled yeast telomeric oligonucleotide CACCACACCCACACACCACA (SEQ ID NO.: 37)

5   (Counter, C.M., *et al.*, *Embo. J. 11*, 1921-9 (1992)). ·

Tetrad analysis: Diploid derivatives of the transposon-mutagenized yeast lacking the pYCPR+ plasmid were sporulated and tetrads were dissected according to standard methods (Guthrie, C., & Fink, G.R., *Guide to yeast*

10   *genetics and molecular biology* (Academic Press, New York, 1991)). Presence of the *rad52::HIS3* allele and the *miniTn3::lacZ::LEU2* insertions were assayed on -His and -Leu synthetic complete plates, respectively.

Yeast extracts: 6 litres of yeast cultures were

15   harvested at an optical density of 0.4-0.6 at 600 nm, resuspended in TMG buffer, disrupted with a bead beater and centrifuged for 90' at 100,000*g* at 4°C as described (Cohn, M. & Blackburn, E.H., *Science 269*, 396-400 (1995)). The supernatant was decanted, flash frozen and stored at ~70°C.

20   Extracts typically had a protein concentration of 16-20 mg/ml.

Telomerase assay: Telomerase activity was assayed essentially as outlined by Cohn and Blackburn with the following two modifications: first, 4 μg of crude S100

25   yeast extract was incubated with the oligonucleotide TGTCTGGGTGTCTGGG (SEQ ID NO.: 38) and second, the reaction products were purified and resolved on 15% acrylamide, 7M urea sequencing gels (Counter, C.M. *et al.*, *Proc. Natl. Acad. Sci. USA 91*, 2900-4 (1994)).

30   Epitope tagging: An *HA-URA3-HA* cassette was introduced into the 3' end of the *EST2* gene by homologous recombination, and then counter-selected on FOA to excise

-37-

the *URA3* gene and generate three copies of the influenza

hemagglutinin tag in frame with *EST2* (Schneider, B.L. *et*

al., Yeast 11, 1265-74 (1995)). The construction of this

epitope-tagged version deleted the 10 carboxy-terminal

5   amino acids of Est2; these codons contain a TA repeat

sequence that is present in multiple copies in the yeast

genome.

      Immunoprecipitation and immunodepletion: 540 µg of

yeast extract was diluted in 170 µl of TMG buffer (Cohn, M.

10 & Blackburn, E.H., *Science 269*, 396-400 (1995))

supplemented with 150 mM NaCl (buffer TMGN) and incubated

at 4°C with 20 µl of packed protein A-agarose beads pre-

loaded with anti-HA antibody (monoclonal antibody 12CA5).

After 2 hours, the mixture was briefly centrifuged to

15 separate the agarose beads from the supernatant extract.

The beads were then washed four times with buffer TMGN and

resuspended in 50 µl TMGN. As a control, a duplicate

extract was diluted and left untreated on ice for 2 hours.

4 µg of supernatant or untreated extracts and 10 µl of

20 beads were assayed for telomerase activity.

Human Telomerase Catalytic Subunit Gene and Encoded Protein

     The findings presented herein support modulation of

hEST2 (hTERT) RNA expression levels as an important

25 mechanism used in a variety of developmental contexts to

determine the amount of telomerase activity present in

specific cell lineages. Indeed, the levels of the

catalytic subunit of telomerase may represent a

rate-limiting determinant of enzyme activity in many types

30 of cells. Moreover, up-regulation of *hEST2* message may be

an important mechanism through which telomerase becomes

activated during both cellular immortalization and the

progression of malignant tumors.

-38-

hEST2, (also referred to as hTERT) the human telomerase subunit described here, shares extensive sequence similarities with the catalytic subunits of the yeast and ciliate telomerase enzymes. The amino acid

5   sequence conservation between these three enzymes is extensive and is scattered throughout their reading frames. In addition, no related genes were detected in the human genome by Southern blotting analysis. Together, these data support the conclusion that the gene described here encodes

10   the catalytic subunit of the human telomerase holoenzyme. Further proof that this is the case can be obtained using known methods, such as experiments analogous to those performed with the yeast *EST2* gene, described herein and by Lingner and co-workers. (Lingner *et al.*, *Science 276*:561-

15   567 (1997)). For example, experiments that will provide additional proof that the gene described herein encodes the human telomerase subunit include those that demonstrate that mice lacking the mouse *mEST2* homologue also lack telomerase activity; that the hEST2 protein is physically

20   associated with a ribonucleoprotein complex that exhibits telomerase activity; and that alteration of critical residues in the domain of hEST2 that is homologous to RTs inactivates its catalytic function.

As described herein, three telomerases described to

25   date (hEst2, yeast Est2 and Euplotes p123) include motifs that indicate they are distant homologues of a variety of RTs. They also share several unique, telomerase-specific sequence motifs. Yeast, ciliates and mammals represent highly diverged branches of the phylogenetic tree, which

30   suggests that the catalytic subunit of the telomerase was developed early in eukaryotic evolution. It is possible that it was present in the cell that became ancestral to all contemporary eukaryotes. Even earlier, it appears that

-39-

this enzyme shares ancestry with the precursors of the RTs specified by a variety of transposons and viruses.

The repression of *hEST2* mRNA in telomerase-negative cells and tissues and its upregulation that is associated

5   with a number of human tumor cell lines and primary human tumors suggest one mechanism by which telomerase activity might be modulated. During development, the expression of the *hEST2* mRNA may be repressed in many post-embryonic cell lineages, depriving cells in these lineages of the

10   telomerase catalytic subunit. This in turn may underlie the observed progressive telomeric shortening associated with aging in the cells in many of these lineages.

In contrast, the reappearance of telomerase enzyme activity when transformed cells escape from crisis or when

15   tumors progress toward malignancy may, in many cases, be explained mechanistically by the de-repression of *hEST2* mRNA expression. As shown here, in cell cultures, this de-repression occurs in both transformed embryonic kidney cells and lymphocytes when they emerge from crisis and

20   begin to exhibit telomerase activity.   Not addressed here is whether the enhanced *hEST2* RNA expression is achieved at the transcriptional or post-transcriptional level.

Described here is a correlation between *hEST2* mRNA levels and assayable telomerase activity. These two

25   manifestations of *hEST2* gene expression are present in a constant, predictable ratio. This provides the basis for assessing cells for *hEST2* mRNA as an indicator of telomerase activity or assessing cells for telomerase activity as an indicator of *hEST2* mRNA occurrence or level.

30   It is possible that other mechanisms besides the presently observed modulation of *hEST2* mRNA levels may intervene to modulate telomerase activity.

-40-

As described in Example 8, over expression of *hEST2* in
previously (normally) telomerase-negative cells is
sufficient to impart telomerase activity to these cells,
providing clear proof that hEST2 is the human telomerase
5   catalytic subunit. The data demonstrate that the ectopic
expression of hTERT in otherwise telomerase-negative human
cells is necessary and sufficient for induction of
telomerase activity. In addition, the physical association
of hTERT with telomerase activity confirms that hTERT is a
10 telomerase subunit. Up-regulation of the *hTERT* gene is the
sole barrier to activation of telomerase in the tested
cells.

The identification of hEST2 as the gene specifying the
telomerase catalytic subunit provides an entree into
15 understanding one of the essential steps in human tumor
pathogenesis -- that leading to cell immortalization.
Enzymatic assays have demonstrated that telomerase is often
activated at a relatively late step in tumor progression
(Harley *et al.*, *Nature 345*:458-460 (1994)). This step may
20 occur when evolving, pre-malignant cell clones have
surmounted the senescence barrier and subsequently, having
exhausted their telomeric ends, encounter the successive
barrier of crisis. At this stage, the activation of
telomerase may enable cells to breach these barriers to
25 further clonal expansion, thereby conferring great
selective advantage on the rare cell that has acquired the
ability to resurrect the long-repressed telomerase
activity. Indeed, activation of telomerase may represent
an essential step in tumor progression.
30 Such dependence on telomerase activity means that this
enzyme represents an attractive target of drugs designed to
interfere with malignant cell proliferation. The finding
that *hEST2* message is up-regulated in human tumors and in

immortalized cells lends further credence to this idea.
Furthermore, the identification of hEST2 as the candidate
telomerase catalytic subunit provides a biochemical reagent
for identifying such drugs.

5   Described herein is a human gene, *hEST2*, which is a
telomerase catalytic subunit gene. The gene transcript
(mRNA) is expressed in primary human tumors, cancer cell
lines and telomerase-positive tissues, but is undetectable,
using the assays described herein, in cell lines known to
10   be telomerase negative and in differentiated telomerase-
negative tissues. The *hEST2* gene shares significant
sequence similarity with a yeast telomerase catalytic
subunit gene (S. cerevisiae EST2) and a ciliate telomerase
catalytic subunit gene (Euplotes p123), as shown in Figure
15   2. The three telomerase enzymes are members of the reverse
transcriptase family of enzymes. There are seven conserved
sequence motifs which define the polymerase domains of
reverse transcriptases and six of these domains are present
in hEST2, including the invariant aspartic acid residues
20   which are required for telomerase activity. Also the
subject of the present invention is DNA which encodes hEST2
protein of SEQ ID NO.: 3; DNA which is the complement of
DNA of SEQ ID NO.: 35; DNA which hybridizes to the
complement of DNA of SEQ ID NO.: 35; DNA which hybridizes
25   to the complement of DNA of SEQ ID NO.: 35 and encodes
hEST2 protein of SEQ ID NO.: 3, ·DNA which is a gene which
localizes to human chromosome 5, subband 5p15.33. A RNA
transcript or RNA message encoded by hEST2 DNA of SEQ ID
NO.: 35 or by DNA which encodes hEST2 protein of SEQ ID
30   NO.: 3 is also the subject of this invention.

As shown in Figure 2, conceptual translation of the
4030 bp *hEST2* DNA shows an open reading frame of 1132 amino
acids which is predicted to be an approximately 127 kDa

●   ●

-42-

protein. As also shown in Figure 2, there is a clear
relatedness among hEST2, yeast EST2 and Euplotes p123 in
regions of the gene outside the RT motifs, particularly
sequence identities in a region just before motif 1 of the
5   hEST2 protein. This region of similarity appears unique to
telomerases and, thus, can be used to produce reagents,
such as antibodies reactive with the unique region and
nucleic acid probes and primers useful to identify *hEST2*
DNA, using, for example, hybridization or amplification
10   methods. *hEST2* is a single-copy gene which is
approximately 40 kb in size and was shown to localize (to
be present on) chromosome 5, particularly subband 5p15.33.
(See Figure 4) Expression levels in normal human tissues
and human cancer cell lines were assessed, as described in
15   Example 3. This assessment revealed two major RNA species
(4.4 kb and 9.5 kb) and one minor RNA species
(approximately 6 kb). *hEST* message was detectable in
several normal tissues, including thymus, testis and
intestine; the latter two tissues are known to be
20   telomerase-positive and the telomerase status of thymus has
not been reported. Using the assays described herein,
hEST2 transcript was not detected in most other normal
human tissues, including heart, brain, placenta, liver,
skeletal muscle and prostate, all of which have been
25   reported to be telomerase-negative.
As described herein, the *hEST2* transcript or message
(mRNA) is expressed in primary human tumors and cancer cell
lines, but is not detectable in telomerase-negative cell
lines and differentiated (normal, nontumorigenic)
30   telomerase-negative tissues. Further, *hEST2* message is not
detectable in pre-crisis, telomerase-negative transformed
cells, but is readily detectable in post-crisis,
telomerase-positive immortalized cells. The work described

herein provides a method of differentiating between
telomerase-positive cells and telomerase-negative cells, as
well as for monitoring a change in telomerase content of
cells. As a result, as described below, the present work
5   provides a method of identifying cells which are telomerase
positive and, thus, of identifying cells which are tumor or
cancer cells or are en route to becoming tumor cells
(malignant).

A method of identifying telomerase-positive cells is
10   the subject of the present invention. Also the subject of
the present invention is a method of identifying cells
which are transformed, malignant, cancerous, tumor or post-
crisis cells or are en route to or likely to become
transformed, malignant, cancerous, tumor or post-crisis
15   cells. For convenience, the term malignant or malignancy
is used herein to refer to transformed, cancer, cancerous,
tumor, tumorigenic and post-crisis cells. For example, the
phrase "a method of identifying malignant cells in an
individual" encompasses a method of identifying cancer
20   cells, transformed cells, tumor cells and/or post-crisis
cells in an individual. The present invention, thus,
provides a method of diagnosing, aiding in the diagnosis of
or predicting an increased likelihood of the occurrence of
cancer, tumor formation and/or the development of
25   malignancy in an individual, particularly a mammal and,
specifically, a human. In the method, telomerase content
of cells is assessed by detecting or measuring hEST2 DNA,
hEST2 transcript (hEST2 message) or hEST2-encoded protein
(hEST2 protein). The quantity of hEST2 DNA, hEST2
30   transcript or hEST2 protein can be determined;
alternatively, the occurrence (presence or absence) of
hEST2 DNA, hEST2 transcript or hEST2 protein can be
detected. The presence of hEST2 DNA, hEST2 message and/or

-44-

hEST2 protein in cells is indicative of the presence of malignant cells or malignancy in the individual.

In one embodiment, *hEST2* DNA, *hEST2* RNA (or both) is detected or measured in tissue or cells obtained from an

5 individual. This can be carried out using known methods, such as hybridization (e.g., *in situ* hybridization) or amplification methods. All or a portion of *hEST2* DNA or RNA can be used in such a method as a probe (e.g., to detect *hEST2* DNA or RNA) or a primer (e.g., to amplify

10 *hEST2* DNA).

As described herein, up-regulation of *hEST2* RNA is associated with activation of telomerase during cell immortalization and may be an important mechanism through which telomerase becomes activated during cellular

15 immortalization, the progression of malignant tumors or both. Thus, detection and/or measurement of *hEST2* RNA provides a means of assessing cells (e.g., mammalian, such as human cells) for malignancy or the likelihood of progression to malignancy. For example, detection of *hEST2*

20 RNA, even without quantification of *hEST2* RNA levels, in cells indicates that telomerase is activated and that the cells are malignant or have an increased likelihood of progression to malignancy (relative to cells in which *hEST2* RNA is not detected). Quantification of *hEST2* RNA can be

25 carried out as well. Higher concentrations of *hEST2* RNA cells are expected to indicate a more advanced stage in malignancy or a greater likelihood of progression to malignancy than is the case with lower *hEST2* RNA levels in cells. *hEST2* RNA levels determined by assessing an

30 individual's cells can be compared with pre-established *hEST2* RNA levels. For example, *hEST2* RNA levels determined

-45-

for an individual's cells can be compared with hEST2 RNA
levels in cells known to be malignant and/or at established
stages of malignancy (e.g., a pre-established reference or
standard).

5   Alternatively, the levels of hEST2 RNA determined for
an individual can be compared with hEST2 RNA levels
determined for the same individual in prior assessments, in
which case the individual serves as his or her own standard
or reference. In this approach, it is possible to monitor
10   (assess over time) the status of the individual. This is
useful, for example, to assess or aid in assessing whether
the malignancy or cancer is progressing, remaining
unchanged or regressing in that individual. This is
particularly useful in assessing the status of an
15   individual's malignancy or cancer before, during and after
therapy (e.g., chemotherapy, radiation, surgery) and to
assess the therapeutic value or suitability of a drug(s) or
other agent(s) for a specific individual in need of
treatment. As mentioned, assessment of hEST2 RNA (or DNA)
20   can be carried out using known methods, such as in situ
hybridization, Rnase protection assay, reverse
transcriptase (RT) PCR, Southern blot analysis or Northern
blot analysis.
        In one embodiment, the presence of malignancy or an
25   increased likelihood of malignancy is assessed in a method
in which a nucleic acid (e.g., DNA) anneals to nucleic acid
(e.g., RNA). For example, the method can be Northern blot
analysis, Rnase protection assay or RT PCR. One embodiment
is as follows: Cells to be assessed for hEST2 DNA or RNA
30   are obtained from the individual and processed to render
DNA and/or RNA in the cells available for annealing with or
hybridization of complementary nucleic acid sequences DNA
or RNA (e.g., with complementary poly- or oligonucleotides

-46-

(DNA or RNA)). All or a portion of hEST2 DNA or hEST2 RNA can be used as the hybridization probe or sequence. As described herein, hEST2 protein shares sequence similarities and identities with yeast p123 and Euplotes

5 Est2p, particularly in a region just before motif 1 (Figure 2, boxed region). As described, this region appears to be unique to telomerases. Thus, it is useful in assays which identify and/or quantify telomerase in cells and provide an assessment of malignancy or the likelihood of progression

10 to malignancy of cells (e.g., from an individual in whom a diagnosis of malignancy or assessment of the likelihood of the occurrence of malignancy is needed). DNA or RNA comprising DNA or RNA encoding region(s) of similarity and/or identify with yeast p123 and/or Euplotes Est2p such

15 as region(s) unique to telomerase, can be used in the hybridization assay or other assay carried out to detect or measure hEST2 DNA or RNA in an individual's cells.

Alternatively, DNA or RNA comprising DNA or RNA which encodes a region or regions unique to hEST2 protein can be

20 used as the probe. In an alternative approach, the hEST2 DNA or RNA is used as a primer in an amplification method. The cells processed to render DNA and/or RNA available for annealing (hybridization) with complementary poly- or oligonucleotides (processed cells) are combined with all or

25 a portion of hEST2 DNA or RNA under conditions appropriate for hybridization of complementary nucleic acids (DNA, RNA) to occur. Whether annealing (hybridization) occurs is then determined. If hybridization occurs (forming complexes of cellular DNA or RNA and the hEST2 poly- or

30 oligonucleotides), it is an indication that the cells contain hEST2 DNA or hEST2 RNA. Poly- or oligonucleotides used in the method can be, for example, DNA which encodes the telomerase motif described herein (e.g., DNA which

-47-

encodes amino acid residues 556 to 565 of SEQ ID NO.: 3 or
amino acid residues 560 to 565 of SEQ ID NO.: 3) or amino
acid residues 1 to 50 of hEST2 protein or a portion
thereof. If *hEST2* RNA is upregulated (expressed), it is
5   indicative of activation of telomerase and of malignancy or
an increased likelihood of progression to malignancy. The
amount of *hEST2* RNA can also be determined (by determining,
for example, the extent to which hybridization with *hEST2*
DNA or RNA occurs) and used to assess the extent of
10 telomerase activity and the stage of malignancy. This
detection and/or measurement of *hEST2* DNA or RNA can be
carried out at various intervals over time to assess the
status of the malignancy (e.g., progression, reversal).

In another embodiment of assessing telomerase content
15 and, thus, malignancy or cancer of cells, hEST2 protein is
analyzed (detected and/or quantified). This can be done
using known methods, such as enzymatic assays or
immunoassays, which indicate hEST2 protein is present in
cells assessed and, optionally, quantify the protein. For
20 example, hEST2 telomerase activity can be assessed by
determining whether extension of a telomeric primer occurs
when a sample (e.g., cells, cell fractions or cell
component(s)) from an individual is combined with the
telomeric primer under conditions appropriate for hEST2
25 protein telomerase activity and telomeric extension.
Alternatively, antibodies which recognize (bind) hEST2
protein can be used to determine if the protein is present
in cells or other sample obtained from an individual. In
the method, cells are obtained from an individual and
30 processed or treated to render proteins in the cells
available for binding with antibodies, thus producing
processed cells. The processed cells are combined or
contacted with antibodies which bind hEST2 protein and

whether binding occurs between antibodies which bind hEST2
protein (hEST2 protein-binding antibodies) and protein in
the cells is determined. If hEST2 protein-binding
antibody/protein binding occurs (to form complexes), it is

5 indicative of the presence of hEST2 protein in the cells
and, thus, of malignancy or an increased likelihood of
progression to malignancy in the individual (in cells of
the individual). The antibodies can be monoclonal
antibodies or polyclonal antibodies (e.g., polyclonal sera)

10 and can be specific for (bind or recognize only) hEST2
protein or can be nonspecific for hEST2 protein (bind or
recognize hEST2 protein and other protein(s)). Humanized
antibodies can also be used. Antibodies which recognize a
motif or epitope unique to hEST2 and/or to members of the

15 class to which hEST2 belongs are particularly useful. For
example, antibodies which specifically bind the telomerase
motif, represented in Figure 2, which is common to hEST2
protein, yeast p123 protein and/or Euplotes EST2 can be
produced, using known methods, and used to assess cells for

20 hEST2 protein. Alternatively, antibodies which
specifically bind a motif which is unique to hEST2 protein
(e.g., all or a portion of amino acid residues 1 to 50 of
hEST2 protein) can be used. As discussed above with
reference to *hEST2* DNA and *hEST2* RNA, the assessment can be

25 detection (determination of presence or absence) or
measurement (quantification). The presence of hEST2
protein is indicative of malignancy or at least of an
increased likelihood of progression to malignancy.

Antibodies which bind or recognize (specifically or

30 non-specifically) hEST2 protein are also a subject of the
present invention. Nucleic acid probes and primers (poly-
or oligonucleotides) comprising *hEST2* DNA or RNA are also

the subject of this invention. The poly- or
oligonucleotides will vary in length and need to be of

sufficient length to bind to and remain bound to *hEST2* DNA
or *hEST2* RNA under the conditions used. They will
generally be at least four to six bases in length and can
comprise the entire *hEST2* DNA or *hEST2* RNA (alone or with

5   additional non *hEST2* DNA or RNA). Preferably the probes or
primers will hybridize to at least a characteristic portion
of *hEST2* DNA or *hEST2* RNA (a portion which is present in
members of the class to which hEST2 belongs), thus making
it possible to identify *hEST2* DNA or *hEST2* RNA

10   substantially to the exclusion of other proteins. For
example, a probe or primer can comprise DNA or RNA which
encodes a characteristic motif or region of hEST2 protein,
such as the telomerase motif (e.g., amino acids 556 to 565
or 560 to 565) or the amino terminal amino acid sequence of

15   hEST2 protein not present in Est2p or p123. (See Figure 2).
For example, a probe or primer encoding amino acid residues
1 to 50 (inclusive) of hEST2 protein (SEQ ID NO.: 3) or a
portion thereof can be used.

Methods of altering *hEST2* DNA transcription and

20   expression, methods of altering hEST2 protein function and
methods of identifying agents which alter (enhance or
reduce) transcription, expression or function are also the
subject of this invention. Also the subject of the present
invention are a method of increasing or shortening the

25   lifespan of cells in culture, *ex vivo* or *in vivo*; agents or
drugs(DNA, RNA, drugs, small organic molecules, enzymes,
for example) useful for lengthening or shortening cell
lifespan and methods of identifying agents which enhance
lifespan. For example, one embodiment of a method of

30   inhibiting hEST2 protein comprises introducing into cells
an agent which inhibits hEST2 protein, directly or
indirectly; as a result, function of the enzyme is

-50-

abolished or the enzyme is inactivated. hEST2 protein is
inhibited directly, for example, by introducing into cells
an agent which binds to or otherwise "ties up" hEST2
protein such that it is less active or inactive (cellular
5   telomerase activity is reduced or eliminated).
Alternatively, a drug or agent which inactivates hEST2 or
inhibits hEST2 catalytic function (and concomitantly
reduces or eliminates telomerase activity) by degrading it
or preventing it from being produced can be introduced into
10   cells in which hEST2 protein is to be inhibited. Such
drugs or agents can be, for example, a small organic
molecule or a dominant negative protein. Agents which
prevent production of *hEST2* message or its further

processing (e.g., to produce DNA) or a dominant negative
15   form of the telomerase protein can also be introduced into
cells to inhibit hEST2 protein. Methods and agents which
inhibit hEST2 catalytic function are useful for treatment
of cancer and, thus, are useful as anti-cancer therapies.
If *hEST2* DNA, *hEST2* RNA or hEST2 protein is inhibited

20   (partially or totally) in cells, lifespan of the cells will
be shortened (shorter than it would be if the DNA, RNA or
protein were not inhibited). For example, if a cell is
malignant or more likely to progress to malignancy because
of hEST2 function, an agent or drug which inhibits *hEST2*

25   transcription or expression or hEST2 protein function in
the cell will also shorten the lifespan of the cell because
telomerase activity (and, thus, chromosomal extension or
maintenance) will be inhibited. The agent is useful as a
therapeutic for treating or preventing malignancy in
30   individuals in whom malignant cells or cells with increased
likelihood to progress to malignancy are present. These
agents include, for example, enzyme (telomerase or hEST2
protein) inhibitors, enzymes which degrade hEST2 protein,

hEST2 transcriptional regulators, antisense molecules
(e.g., DNA, RNA, PNA) and dominant negative mutant forms of
hEST2. In a method of the present invention in which
lifespan of cells is altered (increased or decreased) in an
5   individual, a drug or agent is introduced into the
individual in such a manner that it enters cells of the
individual in sufficient quantity to have the desired
effect (increase or decrease in cell lifespan). For
example, a drug which prevents production of hEST2
10   transcript of hEST2 protein function (directly or
indirectly) is introduced into an individual, using known
methods, in sufficient quantities to enter cells, such as a
tumor, precancerous or cancerous cells, whose lifespan is
to be decreased (e.g., made shorter than would be the case
15   in the absence of the drug). Alternatively, a drug which
enhances cell lifespan can be introduced into an individual
in such a manner that it enters cells in sufficient
quantity to enhance hEST2 protein expression or prolong its
activity (e.g., by blocking its degradation).
20   The present invention is also a method of enhancing or
increasing the lifespan of cells in culture, in which
telomerase activity or function is enhanced, such as by
introducing hEST2 DNA or hEST2 protein into the cells or by
activating an endogenous hEST2 gene. For example, the
25   lifespan of normal human or other mammalian cells can be
extended (immortalized human or other mammalian cells can
be produced) and the resulting cells used for therapeutic
purposes (e.g., grafting of tissue (such as skin) or of
organs), screening or assay methods or production of
30   proteins or other cellular products. For example, the
lifespan of epithelial cells, keratinocytes or endothelial
cells can be extended by introducing hEST2 DNA into the
cells, in which the hEST2 DNA is expressed or introducing

hEST2 protein into the cells. The resulting cells with
longer lifespan can be transplanted into or grafted onto an
individual (e.g., as skin grafts, as systems for delivery
of therapeutic proteins, such as hormones and enzymes), to
5   whom they provide therapeutic benefit. In the method of
extending the lifespan of cultured cells, cells whose
lifespan is to be extended are cultured under conditions
appropriate for their viability and *hEST2* DNA or hEST2
protein is introduced into them. The resulting cells are
10 maintained under conditions appropriate for expression of
hEST2 DNA and/or activity of hEST2 protein, with the result
that the lifespan of the cells is enhanced. At an
appropriate time (e.g., after sufficient cells with
enhanced lifespan are available) enhanced lifespan cells
15 (such as keratinocytes) are transplanted into an individual
(e.g., as a skin graft). cells.

The present invention is illustrated by the following
examples, which are not intended to be limiting in any way.

EXAMPLE 1:   Cloning of *hEST2 (hTERT)*
20   The expressed sequence tag database (dbEST) was
searched for sequences related to the yeast protein Est2p
(Lendvay *et al.*, *Genetics 144*:1399-1412 (1996)) and the
Euplotes protein p123 (Lingner *et al.*, *Science 276*:561-567
(1997)), using the program TBLASTN and the server
25 http://www.ncbi.nlm.nih.gov/BLAST/. This resulted in the
identification of a homologous expressed sequence tag,
Genbank accession # AA281296, which derived from Soares
NbHTGBC cDNA clone 712562. DNA from clone plasmid 712562
was PCR-amplified with primers HT-1 (5'
30 -AAGTTCCTGCACTGGCTGATGAG- 3')(SEQ ID NO.: 39) and HT-5 (5'
-TCGTAGTTGAGCACGCTGAACAG- 3')(SEQ ID NO.: 40). The

-53-

resulting 377 bp fragment was used to probe 1 ZAP phage
cDNA libraries derived from the human Jurkat T-cell
lymphoma (Stratagene, La Jolla, CA) and human Nalm-6 pre-B
cell leukemia cell lines (Weissbach et al., J. Biol. Chem.
5   269:20517-20521 (1994)). A total of seven independent
clones were isolated from these two libraries. Three of
these cDNA clones together with plasmid 712562 were
sequenced completely in both directions. The remaining
clones were sequenced in specific regions. Reprobing of
10   the Jurkat library with the 5'-most 500 bp region
identified from the hEST2 cDNA clones yielded one new clone
containing an insert that overlapped with already
determined sequences.

Rapid amplification of cDNA ends (RACE) was performed
15   by PCR amplifying testis Marathon-ready cDNA (Clontech,
Palo Alto, CA) with flanking primer AP-1 (Clontech) and
hEST2 primer R0096 (5' -CAAGAAACCCACGGTCACTCGGTCCACGCG-
3')(SEQ ID NO.: 41), and then re-amplifying with flanking
primer AP-2 and hEST2 primer R0098 (5'
20   -CAGCTCCTTCAGGCAGGACACCTGCGGG- 3')(SEQ ID NO.: 42). The
product was subcloned and sequenced in both directions.
RACE was also performed on aliquoted pools of plasmid DNA
from a human testis cDNA library (Reduced Complexity cDNA
Analysis, Qingyun Liu and Fang Chen, unpublished results)
25   with primers HT-21 (5' -CAGGTGACACCACACAGAAA- 3') (SEQ ID
NO.: 43) or HT-22 (5' -TTCCAAGCAGCTCCAGAAA- 3')(SEQ ID NO.:
44) and a vector primer, re-amplified with the vector
primer and hEST2 primers R0098 or R0097 (5'
-CCTTCGGGGTCCACTAGCGTGTGGCGG)(SEQ ID NO.: 45), then
30   purified and sequenced.

DNA sequencing reactions were performed with the
AmpliTaq FS Prism ready reaction cycle sequencing kit
(Perkin-Elmer/ABI) and electrophoresed on a 373 A Stretch

-54-

ABI DNA sequencer. The resulting sequences were assembled
into a contig using the program Sequencher 3.0 (Gene Codes
Corporation, Ann Arbor, MI). The 1132 amino acid ORF
identified in this contig was aligned with p123 and Est2p
5   using the Pattern-Induced Multi-sequence Alignment program
version 1.4 (R. F. Smith, Baylor College of Medicine & T.
F. Smith, Boston University) using the server
http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.h
tml, with minor modifications to the final alignment.
10   BLASTP searches were performed against the non-redundant
protein databases using the server
http://www.ncbi.nlm.nih.gov/BLAST/.

EXAMPLE 2:   Identification of the *hEST2* Gene

The sequences derived from the catalytic subunit of
15   the *S. cerevisiae* telomerase, Est2p, and from the Euplotes
p123 protein (Lendvay *et al.*, *Genetics 144*:1399-1412
(1996); Counter *et al.*, *Proc. Natl. Acad. Sci.*, *USA
94*:9202-9207 (1997); Lingner *et al.*, *Science 276*:561-567
(1997)), were used to identify a human homologue (Genbank
20   accession # AA281296) in the National Center for
Biotechnology Information expressed sequence tag database.
As shown in Figure 2 (underlined sequence), the conceptual
translation of this DNA sequence shows clear relatedness to
both the yeast and ciliate telomerase sequences as
25   evidenced by identical or similar amino acid residues
scattered throughout the entire length of the expressed
sequence tag.
The identified expressed sequence tag provided only a
fragment of the putative human telomerase open reading
30   frame. Therefore, a human Jurkat T-cell lymphoma and a
human Nalm-6 pre-B cell leukemia cDNA library were screened

-55-

with a probe derived from the expressed sequence tag.
Seven cDNA clones were retrieved. The resulting sequence
was extended further in the 5' direction by rapid
amplification of cDNA ends (RACE) on human testis cDNA and
5   on an independently generated human testis cDNA library.
Assembly of the cDNA clones and RACE products,
together with the clone containing the expressed sequence
tag, resulted in a contiguous sequence spanning 4030 bp.
Conceptual translation of this 4 kbp sequence reveals an
10 open reading frame of 1132 amino acids which is predicted
to encode a 127 kDa protein (Figure 2). Although we have
not identified an in-frame upstream stop codon in the
sequence, the first methionine identified by RACE (Figure
2) is a candidate for the translation start site, as the
15 sequence of this putative translation initiation site,
CCCGCGAUGC (SEQ ID NO.: 46), is similar to the consensus
GCC(A/G)CCAUGG (SEQ ID NO.: 47) characteristic of
translation initiation sites (Kozak, *Cell* 44:238-292
(1986)).
20   The predicted 127 kDa protein shares extensive
sequence similarity with the entire sequences of the
Euplotes and yeast telomerase subunits (Figure 2) and
extends beyond the amino- and carboxyl-termini of these
proteins. A BLAST search reveals that the probabilities of
25 these similarities occurring by chance are 1.3 x 10^{-18} and 3
x 10^{-13}, respectively. By way of comparison, the
probability of similarity between the yeast and Euplotes
telomerases in a protein BLAST search is 6.9 x 10^{-6}. The
human gene was initially named *hEST2* (*human EST2* homologue)
30 to reflect its clear relationship with the yeast gene, the
first of these genes to be described. The current name for
the gene is *hTERT*, which stands for human TElomerase
Reverse Transcriptase. *EST2* was named because of the

phenotype of ever shortening telomeres caused by its mutant
alleles (Lendvay et al., Genetics 144:1399-1412 (1996)) and
was later demonstrated to encode the yeast telomerase
catalytic subunit (Counter et al., Proc. Natl. Acad. Sci.,
5   USA 94:9202-9207 (1997); Lingner et al., Science 276:561-
567 (1997)).

Like the yeast and ciliate telomerase proteins, hEST2
(hTERT) is a member of the reverse transcriptase (RT) family
of enzymes (Figures 2 and 3A-3F). Seven conserved sequence
10 motifs, which define the polymerase domains of these
enzymes, are shared among the otherwise highly divergent RT
family (Poch et al., EMBO J. 8:3867-3874 (1989); Xiong and
Eickbush, EMBO J. 9:3353-3362 (1990)). p123 and Est2p
share six of these motifs with, most prominently, the a2-Sc
15 enzyme, an RT that is encoded within the second intron of
the yeast COX1 gene (Kennell et al., Cell 73:133-146
(1993)). These six motifs, including the invariant
aspartic acid residues known to be required for telomerase
enzymatic function (Counter et al., Proc. Natl. Acad. Sci.,
20 USA 94:9202-9207 (1997); Lingner et al., Science 276:561-
567 (1997)), are found at the appropriate positions of the
predicted sequence of hEST2 (Figures 2 and 3A-3F). Thus,
the proposed human telomerase catalytic subunit, like its
yeast and ciliate counterparts, belongs to the RT
25 superfamily of enzymes.

Although hEST2 shares some sequence similarity with
RTs, it is not a conventional RT. Rather, it is far more
closely related to the telomerase catalytic subunits of
yeast and ciliates than to other RTs. Whereas the BLAST
30 probability of sequence similarity between hEST2 and the
telomerase subunits of the single-cell eukaryotes arising
by chance is 10^{-13} to 10^{-18}, the chance probability of

sequence similarity with the next most closely related RT, a2-Sc, is 0.12. Beyond the motifs that define the polymerase domains of these various enzymes, hEST2 shows no sequence similarity with RTs. In contrast, in its domains

5 that lie N-terminal to the polymerase domain, BLAST searches identify clear relatedness between hEST2 and both p123 and Est2p, the chance occurrence of these similarities being 1.6×10^{-9} and 1.8×10^{-4} respectively. Many of the sequence identities in the N-termini of hEST2, p123 and

10 Est2p reside in a region just before motif 1 (Figure 2, boxed region). This sequence is not found in RTs, nor is it apparent in other proteins, suggesting the presence of motifs that may be unique to telomerases. Identification of the catalytic subunits of yet other telomerases will be

15 required to validate this possibility.

Even within the hEST2 domain that share sequence similarity with RTs, it is clear that hEST2 is more closely related to the already described telomerases than it is to non-telomerase RTs . For example, the sequence similarity

20 of the region encompassing the RT motifs between hEST2 and the catalytic subunits of yeast and Euplotes has a probability of chance occurrence of 5.7×10^{-6} and 1.9×10^{-5} respectively compared to 0.0056 for a2-Sc, the next most closely related non-telomerase RT. Within the RT

25 motifs are several amino acids that are invariant among the telomerases but divergent between telomerases and non-telomerase RTs, or alternatively nearly invariant among non-telomerase RTs but divergent between these RTs and telomerases (Figures 3A-3F).

30 In summary, hEST2, Est2p, and p123 form a clearly defined subgroup within the RT family. For these reasons, hEST2 is a human homologue and very likely an orthologue of the microbial enzymes described to date.

-58-

Sequencing of a number of cDNA clones has revealed two distinct forms of *hEST2* transcripts. See Figures 5A-5B and Figures 7A-7C. Four independent cDNA clones, isolated from three independently generated libraries deriving from
5 distinct cell types, lack an identical 182 bp segment within the open reading frame. The absence of this segment leads to a shift in reading frame that introduces a premature termination codon. Both forms of the *hEST2* transcript were detected by RT-PCR in a variety of human
10 cell types. Information on the intron-exon boundaries of *hEST2* is not available. The simplest interpretation of these data is that the sequence difference between the two groups of cDNAs reflects the existence of two alternatively spliced mRNAs of the *hEST2* gene. The physiological
15 consequences of the expression of the potential non-functional *hEST2* transcript are obscure at present.

EXAMPLE 3: Chromosomal Localization of *hEST2*

The *hEST2* cDNA was used as a probe in Southern blot analyses of human genomic DNA. These reveal *hEST2* to be a
20 single-copy gene with an estimated size of 40 kb. All the genomic sequences reactive with the cDNA probe appear to be components of this ~40 kb locus, suggesting that there are no other closely related genes in the human genome.

hEST2 was localized to a specific chromosomal region
25 by analyzing two independent panels of hamster-human radiation hybrid (RH) cells with two markers spanning different regions of *hEST2*. Initial mapping using the Genebridge 4 RH panel placed both *hEST2* markers between sequence-tagged sites (STS) WI-9907 and D5S417 (Figure 4).
30 Independent confirmation of this localization was then obtained by mapping carried out with a second panel, the

-59-

Stanford G3 RH panel. This second mapping placed both
hEST2 markers next to STS marker AFMA139YA9 (GDB locus
D5S678) which itself is localized between the
above-mentioned markers WI-9907 and D5S417. These markers
5   are present at the telomeric end of chromosome 5p (Hudson
et al., Science 270:1945-1954 (1995)).

Mapping of the *hEST2* locus was further refined by
localizing it to subband 5p15.33, since the STS markers
D5S678 and D5S417 have been assigned to this band on the
10   Genethon YAC contig map (Chumakov *et al., Nature 377*:175-
297 (1995)). This localization is consistent with
fluorescence in situ hybridization (FISH) analysis of YAC
767E1. This YAC maps further away from the telomere than
D5S678 and has been assigned to chromosome 5p15.33 by FISH
15   (Chumakov *et al., Nature 377*:175-297 (1995)).

Chromosome 5p is one of the most common targets for
amplification in non-small-cell lung cancers; it is
amplified in ~70% of tumors (Balsara *et al., Cancer Res.
56*:645-650 (1997); Petersen *et al., Cancer Res. 57*:2331-
20   *2335* (1997)). The effects of this amplification on *hEST2*
expression levels are unknown.

EXAMPLE 4:   Expression of *hEST2* Transcripts
Telomerase becomes activated during tumor progression.
and, as discussed above, this activation has been
25   associated with the immortalization of tumor cells. A
variety of mechanisms might be invoked to explain such
activation, among which is the induction of the expression
of one or more telomerase subunits. The telomerase
holoenzyme is presumed to exist as a multi-subunit
30   ribonucleoprotein complex and, therefore, the levels of any
one of the subunits, including those of the catalytic

subunit described here, might be rate-limiting in determining enzyme activity. Alternatively, the components of the telomerase holoenzyme might be expressed constitutively and subject to various types of
5   post-translational modification that would govern their activity. As mentioned earlier, transcript levels of the telomerase RNA subunit and of the *TP1/TLP1* gene do not necessarily correlate with telomerase activity (Feng *et al.*, *Science 269*:1236-1241 (1995); Avilion *et al.*, *Cancer*
10 *Res. 56*:645-650 (1996); Blasco *et al.*, *Nat. Genet. 12*:200-204 (1996); Harrington *et al.*, *Science 275*:973-977 (1997); Nakayama *et al.*, *Cell 88*:875-884 (1997)), implying that at least one other mechanism is responsible for regulating human telomerase.

15   The expression levels of *hEST2* mRNA in various cell types were analyzed, using both RNA Northern hybridizations and RNase protection assays, in order to address one of the remaining possible regulatory mechanisms,. RNA blots prepared from a panel of normal human tissues and human
20 cancer cell lines were probed with cDNA fragments deriving from two independent, non-overlapping regions of the *hEST2* gene. This probing revealed two major RNA species migrating near the 4.4 kb and the 9.5 kb markers, as well as a minor species of ~6 kb (Figures 8A-8F). Each of these
25 RNA species was recognized by both probes, confirming that each represents an *hEST2* mRNA.

    hEST2 message was detectable in several normal tissues including thymus, testis, and intestine. Of these, testis (Kim *et al.*, *Science 266*:2011-2015 (1994); Wright *et al.*,
30 *Dev. Genet. 18*:173-179 (1996)) and intestine (Hiyama *et al.*, *Int. J. Oncol. 9*:453-458 (1996)) are known to be

telomerase-positive, while the telomerase status of the thymus has not been reported. In marked contrast, the *hEST2* transcript was undetectable in our assays in most other normal human tissues, including heart, brain,

5 placenta, liver, skeletal muscle, and prostate (Figures 8A 8B, 8D and 8E), all of which have been reported to be telomerase-negative (Kim *et al.*, *Science 266*:2011-2015 (1994); Wright *et al.*, *Dev. Genet. 18*:173-179 (1996); Shay and Bacchetti, *Eur. J. Cancer 33*:787-791 (1997)). The

10 absence of detectable *hEST2* message in ovary may seem paradoxical, as ovary is a germline tissue, and germline tissues have been reported to harbor significant levels of telomerase. However, oocyte division is completed by birth, and while fetal ovary is telomerase-positive (Kim *et*

15 *al.*, *Science 266*:2011-2015 (1994); Wright *et al.*, *Dev. Genet. 18*:173-179 (1996)), both adult ovarian epithelium and mature oocytes are telomerase-negative (Counter *et al.*, *Proc. Natl. Acad. Sci.*, *USA 91*:2900-2904 (1994); Wright *et al.*, *Dev. Genet. 18*:173-179 (1996)). It is also possible

20 that the levels of *hEST2* are below the threshold of detection. The 7 kb band seen in muscle tissue appears to be an artifact, as it was not observed with an independent *hEST2* probe.

In contrast to its absence in the majority of normal

25 tissues, *hEST2* mRNA was strongly expressed in a variety of cancer cell lines, most strikingly in the leukemic cell lines HL-60 and K-562 (Figures 8C and 8F). It is unclear why only very low levels of *hEST2* message were observed in HeLa cells on this particular Northern blot (Figures 8C and

30 8F); reanalysis of independently prepared HeLa cell RNA both by Northern blot and by RNase protection demonstrated

-62-

that *hEST2* mRNA is present in HeLa cells at high levels
comparable to those seen in K-562 cells. The two major and
one minor *hEST2* transcript appear to be expressed in the
same relative proportions in all cell types that yielded
5   detectable *hEST2* mRNA.

EXAMPLE 5: hEST2 Expression in Primary Human Cancers
          The expression of *hEST2* mRNA in cancer cell lines
suggested that *hEST2* transcript levels might be elevated in
primary tumors as well. To determine if this is the case,
10 RNA was extracted from a variety of tumor samples as well
as from normal control tissues and analyzed this for the
presence of *hEST2* mRNA using an RNase protection assay. In
total, 11 of 11 tumor samples examined showed detectable
levels of *hEST2* message (Figures 9A-9D). *hEST2* RNA was
15 undetectable in normal breast and ovarian tissue but was
expressed at significant levels in 2 of 2 breast tumors as
well as 2 of 2 breast tumor-derived cell lines, and in 4 of
4 ovarian tumors.
          As expected from Northern hybridization analysis of
20 *hEST2* RNA (Figures 8A-8F) and the known pattern of
telomerase catalytic activity in human tissues (Kim *et al.,*
Science 266:2011-2015 (1994); Hiyama *et al., Int. J. Oncol.*
9:453-458 (1996)), the *hEST2* transcript was detected at
high levels by the RNase protection assay in testis and at
25 moderate levels in colon. Similarly, using the RNase
protection assay, four colon tumor samples and a testicular
tumor sample were found to express detectable levels of
hEST2 RNA (Figures 9A-9D); two of the colon tumors showed
significantly elevated levels as well. These data suggest
30 that the *hEST2* message is expressed in a very high

percentage of tumors and is specifically induced in tumors
that arise from telomerase-negative tissues.

EXAMPLE 6:   Up-regulation of *hEST2* mRNA Is Associated with
            Telomerase Activation and Cellular Immortalization

5   The strong expression of the *hEST2* message observed in
several tumors and cancer cell lines suggested that the
levels of this transcript are correlated with the amount of
telomerase enzyme activity in these various cell types.   To
investigate this possibility, telomerase activity and *hEST2*

10 RNA level were analyzed in a panel of non-immortalized and
immortalized cell lines.   Using the TRAP telomerase assay
(Kim *et al.*, *Science 266*:2011-2015 (1994)), two mortal
fibroblast strains, WI-38 and IMR-90, were found to lack
detectable telomerase activity.   In contrast, telomerase

15 activity was readily detectable in three immortal cell
lines, HeLa, 293, and K-562.   The telomerase-negative cells
also lacked detectable *hEST2* message as gauged by an RNase
protection assay, while the immortal telomerase-positive
cell lines expressed significant levels of *hEST2* RNA.

20 These results indicate that *hEST2* message levels correlate
closely with telomerase activity.

        Thus, as described herein, *hEST2* RNA expression and
telomerase activity are present in immortal transformed
cells, but absent in mortal normal cells.   These findings

25 support the role of induction of *hEST2* expression in the
activation of telomerase that occurs during cellular
immortalization.   That this is the case was shown by
analyzing *hEST2* transcript levels by RNase protection, and
comparing the levels of hTR and telomerase activity in

30 pre-crisis cells prior to the up-regulation of telomerase,
and in post-crisis telomerase-positive immortal cells from

-64-

two different transformed human cell populations:
Epstein-Barr virus-transformed B lymphocytes (B4 cells) and
SV40-T antigen transformed embryonic kidney cells (HA1
cells). Result observed were consistent with previously
5　reported assessments (Counter *et al.*, *EMBO J.* *11*:1921-1929
(1992), *Proc. Natl. Acad. Sci.*, *USA 91*:2900-2904 (1994);
Avilion *et al.*, *Cancer Res. 56*:645-650 (1996)). Little or
no telomerase activity was detected in cells prior to
crisis when telomere length decreases, but abundant levels
10　were detected in post-crisis cells which maintain telomere
length. In contrast, the levels of *hTR* remained
essentially constant throughout the immortalization
process. The levels of *hEST2* RNA, on the other hand,
parallel telomerase activity during cell immortalization.
15　*hEST2* RNA was undetectable in the pre-crisis cells but was
clearly present in the post-crisis, telomerase-positive
cells. Induction of *hEST2* message is the rate-limiting
step for the activation of telomerase during
immortalization.

20　EXAMPLE 7: Down-regulation of *hEST2* Expression upon
Cellular Differentiation
　　　　The up-regulation of *hEST2* RNA is associated with the
activation of telomerase during cell immortalization. It
remained unclear whether conditions that repress telomerase
25　activity in cultured cells might similarly operate by
shutting down expression of the *hEST2* mRNA in these cells.
Human HL-60 promyelocytic leukemia cells can be induced to
differentiate to mature granulocytes by treatment with
retinoic acid. During this process, telomerase activity
30　has been shown to decline over a period of two to five days
(Sharma *et al.*, *Proc. Natl. Acad. Sci.*, *USA 92*:12343-12346

-65-

(1995); Bestilny et al., Cancer Res. 56:3796-3802 (1996);

Savoysky et al., Biochem. Biophys. Res. Commun. 226:329-334

(1996); Xu et al., Leukemia 10:1354-1357 (1996)).

Induction of HL-60 differentiation was shown to lead

5 to the disappearance of the hEST2 mRNA within 24 hours,

foreshadowing the loss of telomerase activity which

declines more slowly.   Once again, this contrasts with the

behavior of the human telomerase RNA subunit, whose

expression during induced differentiation remains constant,

10 as also previously reported (Bestilny et al., Cancer Res.

56:3796-3802 (1996)). Results presented here show that the

levels of the hEST2 mRNA decline precipitously within the

first three hours after induced differentiation. This

rapid down-modulation of is compatible with a short

15 half-life for the hEST2 message and suggests that the

levels of this RNA species are under tight control in these

cells. The contrasting delay in the decline of telomerase

activity is consistent with the reported ~24 hour

half-life of the enzymatic activity after cycloheximide

20 treatment (Holt et al., Mol. Cell. Biol. 16:2932-2939

(1996)).

EXAMPLE 8 Ectopic Expression of hTERT mRNA in Telomerase-
Negative Cells and Assessment of Expression on Levels of
Telomerase Activity

25   The hTERT cDNA was introduced into a mammalian

expression construct carrying the CMV promoter. To

distinguish the ectopically expressed hTERT protein from

its endogenous counterpart, the C-terminus of the

vector-encoded protein was marked with an influenza virus

30 hemagglutinin (HA) epitope tag, yielding the plasmid

-66-

pCI-neo-*hTERT-HA*. Such a modification does not affect the
catalytic activity of the Est2p protein of *S. cerevisiae*
(Counter, C.M., *et al.*, "The catalytic subunit of yeast
telomerase", *Proc. Natl. Acad. Sci. USA, 94*:9202-9207

5   (1997).

This *hTERT-HA* construct and a control empty vector
were transfected into cells of the SV40-transformed GM847
human fibroblast line (Pereira-Smith, O.M., and Smith,
J.R., "Genetic analysis of indefinite division in human

10   cells: identification of four complementation groups",
Proc. Natl. Acad. Sci. USA, 85:6042-6046 (1988). In
contrast to most immortal human cells, which appear to
activate telomerase in order to maintain telomere length
(Shay, J.W., and Bacchetti, S., "A survey of telomerase

15   activity in human cancer", *Eur. J. Cancer, 33*:787-791
(1997), the immortal GM847 cells are telomerase-negative
(Bryan, T.M., *et al.*, "Telomere elongation in immortal
human cells without detectable telomerase activity", *EMBO
J., 14*:4240-4248 (1995). Moreover, while the *hTR* gene is

20   transcribed in these cells(Bryan, T.M., *et al.*, "The
telomere lengthening mechanism in telomerase-negative
immortal human cells does not involve the telomerase RNA
subunit", *Hum. Mol. Genet., 6*:921-926 (1997), they lack
detectable levels of *hTERT* mRNA (Kilian, A., *et al.*,

25   "Isolation of a candidate human telomerase catalytic
subunit gene, which reveals complex splicing patterns in
different cell types", *Hum. Mol. Genet., 6*:2011-2019
(1997). These observations have led to the conclusion that
GM847 cells employ a telomerase-independent mechanism to

30   maintain telomere length (Murnane, J.P., *et al.*, "Telomere
dynamics in an immortal human cell line", *EMBO J.,*

13:4953-4962 (1994); (Bryan, T.M., *et al.*, "Telomere elongation in immortal human cells without detectable telomerase activity", *EMBO J.*, *14*:4240-4248 (1995), perhaps analogous to the recombination-based pathway used by yeast

5   cells to allow chromosomes to maintain telomeres in the absence of a functional telomerase pathway (Lundblad, V., and Blackburn, E.H., "An alternative pathway for yeast telomere maintenance rescues est1- senescence. Cell *73*, 347-360 (1993). Unlike normal, telomerase-negative human

10 somatic cells, which lack replicative immortality, these GM847 cells could be propagated indefinitely following transfection, allowing us to study the properties of clonally isolated cell populations that have stably acquired the introduced *hTERT* gene.

15   A number of stably transfected GM847 cell clones were generated with either the control vector or the *hTERT-HA* expression vector, mRNA expression of *hTERT* was analyzed by RNase protection using probes that specifically recognize either the transfected *hTERT-HA* mRNA or the endogenous

20 *hTERT* transcript. As expected, *hTERT-HA* transcript was detected only in GM847 sublines stably transfected with an *hTERT-HA* expression plasmid, but not in untransfected telomerase-positive control cell lines 293 and HL-60, in the parental GM847 line or in GM847 sublines transfected

25 with the empty vector. The cells expressing *hTERT-HA* did not express the endogenous *hTERT* transcript, despite the fact that this RNA is clearly detected in telomerase-positive cells. Lastly, in accord with previous observations that the level of the *hTR RNA* subunit of

30 telomerase does not correlate with enzyme activity, this RNA was detected in all cells tested, irrespective of whether the cells had telomerase activity. An actin

control probe demonstrates comparable loading of RNA from
each cell line. Moreover, the specificity of the probes
used was demonstrated by their failure to protect tRNA.

     Expression of hTERT-HA was also analysed at the
5   protein level, by immunoblotting with an anti-HA antibody
probe directed against the HA tag of the vector-encoded
hTERT protein. A ~ 130 kDa product corresponding to the
predicted size of hTERT was detected in those lines derived
from GM847 cells that were stably transfected with the
10 hTERT-HA expression construct, but not in those cell clones
that had been transfected with the empty vector. The
antibody likewise did not detect endogenous (untagged)
hTERT known to be expressed in 293 cells (Meyerson, M., *et
al.* "*hEST2*, the Putative Human Telomerase Catalytic Subunit
15 Gene, Is Up-Regulated in Tumor Cells and during
Immortalization," *Cell*, *90*:785-795 (1997), a telomerase-
positive control cell line.

     Assessment of whether these hitherto telomerase-
negative GM847 cell lines acquired telomerase activity
20 together with the stable ectopic expression of *hTERT* was
carried out. Telomerase activity was measured in these
different cell lines by assaying the ability of a cellular
extract to elongate a primer in a telomerase-specific
manner. The products of this *in vitro* reaction are
.25 subsequently detected by specific PCR amplification,
yielding a ladder of products differing from one another by
6 bp (Kim, N.W., *et al.*, "Specific association of human
telomerase activity with immortal cells and cancer",
Science, *266*:2011-2015 (1994)). Telomerase activity was
30 detected in 293 cells, a cell line with one of the highest
levels of telomerase activity known. This activity was
sensitive to heat treatment of the extract, which
inactivates telomerase (Kim, N.W., *et al.*, "Specific

-69-

association of human telomerase activity with immortal cells and cancer", *Science*, *266*:2011-2015 (1994)). In contrast, almost no telomerase products were detectable following assay of extracts from untransfected GM847 cells
5   or from GM847 sublines stably transfected with the empty control vector. This inability to detect telomerase was not due to the presence of a PCR-inhibiting activity, as an internal control was specifically PCR-amplified, (Kim, N.W., and Wu, F. "Advances in quantification and
10 characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP)", *Nucleic Acids Res.*, *25*: 2595-2597 (1997).

In marked contrast, telomerase activity was readily detectable in those clones of GM847 cells that were stably
15 transfected with the *hTERT-HA* expression vector and this activity was sensitive to heat inactivation. The levels of telomerase activity observed in these transfectants approached those seen in extracts from 293 cells. The restoration of telomerase activity in the cells transfected
20 with *hTERT-HA* was not due to the up-regulation of the endogenous *hTERT* gene, as evidenced by failure to detect the corresponding mRNA in these cells. Thus, ectopic expression of *hTERT* in previously telomerase-negative cells is sufficient to generate telomerase activity at levels
25 comparable to those found in immortalized telomerase-positive cells.

The telomerase activity detected in cells transfected with the *hTERT-HA* expression vector was physically associated with ectopically produced hTERT-HA, confirming
30 that hTERT is, as predicted, a constituent of the telomerase holoenzyme. Telomerase activity could be immunoprecipitated with an anti-HA monoclonal antibody from

extracts of either 293 cells or GM847 cells that ectopically express *hTERT-HA*. The telomerase activity was not immunoprecipitated with an antibody directed against an irrelevant antigen (anti-p53), nor was it

5   immunoprecipitated when either antibody was incubated with extracts prepared from control vector-transfected GM847 cells. Taken together, these data indicate that telomerase activity is specifically co-immunoprecipitated with hTERT-HA.

10   Whether the observed induction of telomerase activity following ectopic hTERT expression was unique to GM847 cells was also determined. This was done by transiently transfecting WI-38 normal human fibroblasts, which lack detectable levels of telomerase activity and *hTERT* message

15   but express the hTR gene (Meyerson, M., *et al.*, "*hEST2*, the Putative Human Telomerase Catalytic Subunit Gene, Is Up-Regulated in Tumor Cells and during Immortalization. Cell *90*, 785-795 (1997), with the *hTERT-HA* expression construct. Since transient transfection of normal human

20   cells is extremely inefficient, the plasmid pGreenLantern-1, which encodes the green fluorescent protein (GFP), was co-transfected with either the *hTERT-HA* expression vector or the control vector. Cells expressing GFP, and hence quite likely the co-transfected plasmid,

25   were sorted by virtue of their fluorescence.

         Extracts derived from both populations of fluorescing cells were assayed for telomerase activity. Whereas fibroblasts transfected with pGreenLantern-1 and pCI-neo vector alone lacked enzymatic activity, those

30   co-transfected with the *hTERT-HA* expression vector were clearly telomerase-positive. Transfection with pCI-neo-*hTERT-HA* of IMR-90 cells, another telomerase-

negative normal human fibroblast cell strain that does not normally express hTERT (Kim, N.W., et al., "Specific association of human telomerase activity with immortal cells and cancer", Science, 266: 2011-2015 (1994);

5 (Meyerson, M., et al.,"hEST2, the Putative Human Telomerase Catalytic Subunit Gene, Is Up-Regulated in Tumor Cells and during Immortalization", Cell, 90: 785-795 (1997), also gave rise to telomerase activity (not shown). Thus, ectopic expression of hTERT in these two types of normal

10 human somatic cells results in readily detectable telomerase activity.

Taken together, these data demonstrate that the ectopic expression of hTERT in otherwise telomerase-negative human cells is both necessary and

15 sufficient for induction of telomerase activity. Furthermore, the physical association of hTERT with telomerase activity confirms that hTERT is a telomerase subunit. The fact that forced expression of hTERT sufficed to impart telomerase activity indicates that levels of hTR

20 mRNA and TP-1 or other still unidentified components of the telomerase holoenzyme are not rate-limiting determinants of telomerase activity in these cells. Up-regulation of the hTERT gene is therefore the sole barrier to activation of telomerase in the tested cells.

25 It still remains to be determined if telomerase activity can be restored in this fashion in all telomerase-negative cells. However, results described here show that activity is conferred by ectopic expression of hTERT in cell types representative of two known classes of

30 telomerase-negative cells: telomerase-negative immortal cell lines and normal mortal human cell strains. Thus, it is likely that expression of hTERT mRNA is the

-72-

rate-determining step for telomerase activation in other
human cells lacking enzyme activity. This makes the
regulation of transcription from the *hTERT* promoter a
potential target for modulation during tumorigenesis and
5   cell immortalization.

Experimental Procedures
        The following methods and materials were used in the
experiments which are described above.

Radiation Hybrid Mapping
10   25 ng of genomic DNA from hybrid clones of the
Genebridge 4 and Stanford G3 radiation hybrid (RH) mapping
panels (Research Genetics, Inc. Huntsville, AL) was
PCR-amplified with the primer pair M1 (forward-5'
-CACAGCCAGGCCGAGAGCAGA- 3'(SEQ ID NO.: 48) and reverse-5'
15   -AGGCCTCAGCCGGACACTCAG- 3')(SEQ ID NO.: 49), yielding a 170
bp fragment in the 3'-untranslated region, and with primer
pair M2 (forward-5' -GAAGAAAACATTTCTGTCGTG- 3'(SEQ ID NO.:
50) and reverse- 5' -GCCCTTGGCCCCCAGCGACAT- 3')(SEQ ID NO.:
51), generating a 180 bp fragment crossing a putative
20   intron-exon boundary near the *hEST2* stop codon. PCR was
carried out for 35 cycles of 94°C for 0.5 min., 69°C for M1
or 65°C for M2 for 0.5 min. and 72°C for 1.5 min. The
results of the PCR screening were analyzed using the
statistical program RHMAP provided through the following
25   two e-mail servers on the World Wide Web:
http://shgc-www.stanford.edu and
http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl.
Linkage of STS markers to a physical chromosomal map was
achieved by accessing the servers:
30   http://www.cephb.fr/ceph-genethon-map.html and
http://www-genome.wi.mit.edu. Comparison to YACs

-73-

physically mapped by FISH was achieved with the server:
ftp://ftp.cephb.fr/pub/ceph-genethon-map/FISH/29MAR95.DAT.

RNA Isolation from Cell Lines and Primary Tissues
        Cell lines were obtained from the American Type
5   Culture Collection and grown under standard conditions.
For HL-60 differentiation assays, cells were pelleted and
resuspended in normal growth medium plus all-*trans* retinoic

acid (Sigma) at a final concentration of 1 μM.
        All primary normal and tumor tissues were obtained
10 from the Massachusetts General Hospital tumor bank.
Tissues were processed in a tissue homogenizer. RNA
samples were prepared in the RNA Stat-60 solution (Tel-Test
"B", Friendswood, Texas) according to the manufacturer's
protocol.

15 Northern Hybridization
        Duplicate filters containing poly A(+)-selected mRNAs
from various human tissues and cell lines (Multiple Tissue
Northern Blots, Clontech, Palo Alto, CA), were incubated
according to the manufacturer's instructions with two
20 independent *hEST2* probes. One probe was derived by

PCR-amplifying plasmid 712562 with primers HT-1 and HT-5 to
generate the 377 bp fragment described above; the second,
more 3' probe was generated from the 1064 bp *Stu* I fragment

of the same plasmid. The duplicate blots were then
25 rehybridized with a β-actin probe. Northern blots for
detection of *hTR* were performed with total RNA and an

hTR-specific probe (Feng *et al.*, *Science 269*:1236-1241

(1995)).
RNAse Protection Analysis
30   Radiolabelled RNA probes for RNAse protection analysis
were synthesized using [α-^{32}P]UTP, T7 RNA polymerase and

-74-

the MAXIscript kit (Ambion, Austin, TX). DNA templates for
probe synthesis were created as follows. For the *hEST2*
probe, the insert from plasmid clone 712562 was subcloned
into pUHD 10-3 (a gift from M. Gossen), and template DNA

5   was synthesized by PCR using a forward plasmid-specific
primer and a reverse primer containing 18bp of *hEST2*
complementary sequence (5'-TCTCTGCGGAAGTTCTG)(SEQ ID NO.:
52) and the T7 promoter sequence. For the β-actin probe,
template DNA was synthesized by PCR on a human β-actin cDNA

10   insert (Clontech). The *hEST2* and the β-actin control
probes were hybridized in the same reaction tube. The *hTR*
probe was synthesized directly from the linearized pGRN83
plasmid (Feng *et al.*, *Science 269*:1236-1241 (1995)).

RNase protection analysis was performed using the

15   HybSpeed RPA kit (Ambion) according to the manufacturer's
protocol. Briefly, sample RNA and radiolabelled RNA probes
were coprecipitated in ethanol, resuspended in
hybridization buffer, hybridized at 68° C, then digested
with RNases A and T1. Samples were then re-precipitated

20   and analyzed on a 6% denaturing polyacrylamide gel.

Telomerase assays
Telomerase repeat amplification protocol (TRAP) assays
were performed as described (Kim *et al.*, *Science 266*:2011-
2015 (1994)), in some cases using the TRAPeze telomerase

25   detection kit (Oncor, Gaithersburg, MD).

EQUIVALENTS
While this invention has been particularly shown and
described with references to preferred embodiments thereof,
it will be understood by those skilled in the art that

30   various changes in form and details may be made therein
without departing from the spirit and scope of the

-75-

invention as defined by the appended claims. Those skilled
in the art will recognize or be able to ascertain using no
more than routine experimentation, many equivalents to the
specific embodiments of the invention described
5   specifically herein. Such equivalents are intended to be
encompassed in the scope of the claims.

-76-

CLAIMS

What is claimed is:

1. Isolated DNA encoding the catalytic subunit of a eukaryotic telomerase holoenzyme.

5 2. Isolated DNA of Claim 1 which encodes the catalytic subunit of a yeast telomerase holoenzyme.

3. Isolated DNA of Claim 2 wherein the DNA is selected from the group consisting of: DNA of SEQ ID NO.: 1; DNA which hybridizes to the complement of SEQ ID NO.: 10 1; and DNA which encodes the amino acid sequence of SEQ ID NO.: 2.

4. Isolated DNA of Claim 1 which encodes the catalytic subunit of a human telomerase holoenzyme.

5. Isolated DNA of Claim 4 wherein the DNA is selected 15 from the group consisting of: DNA comprising the nucleotide sequence of SEQ ID NO.: 35; DNA which encodes hEST2 protein having the amino acid sequence of SEQ ID NO.: 3; and DNA comprising a nucleotide sequence which hybridizes to the complement of SEQ ID 20 NO.: 35.

6. Isolated DNA of Claim 4 which is an isolated gene which localizes to human chromosome subband 5p15.33.

7. Isolated DNA comprising DNA encoding a human telomerase catalytic subunit, wherein the DNA encodes 25 an RNA transcript which is up-regulated in a human tumor.

-77-

8.   Isolated DNA of Claim 7 which is an isolated human
gene which localizes to human chromosome subband
5p15.33.

9.   Isolated DNA of Claim 7 which comprises *hEST2* cDNA of
5   SEQ ID NO.: 35 or DNA which hybridizes to the
complement of *hEST2* cDNA under conditions of high
stringency.

10.   Isolated DNA of Claim 7 wherein the human tumor is
selected from the group consisting of: breast tumors,
10   ovarian tumors, colon tumors and testicular tumors.

11.   Isolated DNA of Claim 7 which encodes a human
telomerase catalytic subunit which comprises
approximately 1132 amino acid residues.

12.   Isolated DNA of Claim 8 wherein the RNA transcript is
15   also up-regulated in a cancer cell line, a telomerase-
positive tissue or both.

13.   Isolated mRNA which encodes a human telomerase
catalytic subunit, wherein expression of the mRNA is
up-regulated in a human tumor.

20 14.   Isolated mRNA of Claim 13 wherein the human tumor is
selected from the group consisting of: breast tumors,
ovarian tumors, colon tumors and testicular tumors.

15.   Isolated mRNA of Claim 13 wherein the mRNA has the
sequence of SEQ ID NO.: 36.

16. A nucleic acid probe comprising DNA unique to *hEST2* DNA, *EST2* DNA and p123 DNA.

17. The nucleic acid probe of Claim 16 comprising DNA which encodes the telomerase motif of hEST2 protein.

5 18. The nucleic acid probe of Claim 17 comprising nucleotides which encode amino acid residues 556 to 565 of SEQ ID NO.: 3 or nucleotides which encode amino acid residues 1 to 50 of hEST2 protein or a portion thereof.

10 19. A method of assessing cells for malignancy or an increased likelihood of progression to malignancy, comprising:
    a) obtaining cells to be assessed from an individual;
15   b) processing cells obtained in a) to render DNA and/or RNA in the cells available for annealing of the DNA and/or RNA with complementary poly- or oligonucleotides, thereby producing processed cells;
20   c) combining processed cells with hEST2 DNA or RNA under conditions appropriate for annealing of DNA and/or RNA with complementary poly- or oligonucleotides, thereby producing a combination,
25 wherein if annealing occurs in the combination produced in c), it is indicative of activation of telomerase and malignancy or increased likelihood of progression to malignancy.

20. The method of Claim 21 wherein the poly- or
30 oligonucleotides are DNA which encodes the telomerase

motif or amino acid residues 1 to 50 of SEQ ID NO.: 3
or a portion thereof.

21. A method of diagnosing or aiding in the diagnosis of
development of malignancy in an individual,
5   comprising:
         a)   obtaining cells from the individual;
         b)   processing the cells obtained in a) to render
              proteins in the cells available for binding with
              antibodies;
10   c)   combining the product of b) with antibodies which
              bind hEST2 protein; and
         d)   determining whether binding occurs between the
              antibodies which bind hEST2 protein and protein
              in the cell,
15   wherein if binding occurs, it is indicative of the
         presence of hEST2 protein in the cells and of
         malignancy or an increased likelihood of development
         of malignancy in the individual.

22. The method of Claim 21 wherein the antibodies which
20   bind hEST2 protein bind the telomerase motif of hEST2
         protein or bind amino acid residues 1 to 50 of SEQ ID
         NO.: 3 or a portion thereof.

23. A method of reducing expression of *hEST2* RNA and hEST2
         protein in cells of an individual, comprising
25   administering to the individual a drug selected from
         the group consisting of: drugs which inhibit or bind
         hEST2 RNA and prevent or reduce production of hEST2
         protein and drugs which inhibit hEST2 protein function
         or activity.

24. The method of Claim 23 wherein the drug is selected
from the group consisting of: small organic molecules;
enzymes which degrade hEST2 protein; enzyme
inhibitors; HEST2 transcriptional regulators;
5 antisense molecules and dominant negative forms of
hEST2 protein.

25. A method of treating cancer in an individual,
comprising administering to the individual a drug
which inhibits or binds *hEST2* RNA and prevents or
10 reduces production of hEST2 protein or a drug which
inhibits hEST2 protein function or activity, under
conditions appropriate for the drug to enter cells of
the individual, whereby the drug inhibits or binds
hEST2 RNA and prevents or reduces production of hEST2
15 protein or inhibits hEST2 protein function or
activity.

26. The method of Claim 25 wherein the drug is selected
from the group consisting of: enzymes which degrade
hEST2 protein; enzyme inhibitors; hEST2
20 transcriptional regulators; antisense molecules and
dominant negative forms of hEST2 protein.

27. A method of increasing lifespan of cells, comprising:
    (a) introducing into the cells *hEST2* DNA, under
        conditions appropriate for expression of *hEST2*
25   DNA, whereby hEST2 protein is expressed or
    (b) introducing hEST2 protein into the cells, whereby
        sufficient hEST2 protein is expressed or present
        in the cells to increase lifespan of the cells.

28. The method of Claim 27, wherein the cells are cells in
culture.

29. A method of decreasing lifespan of cells in an
individual by reducing hEST2 protein function or
5   activity, comprising administering to the individual a
drug selected from the group consisting of:
(a) drugs which inhibit or bind *hEST2* RNA and prevent
or reduce production of hEST2 protein;
(b) drugs which inhibit hEST2 protein function or
10   activity; and
(c) drugs which inhibit or bind *hEST2* DNA and prevent
or reduce production of *hEST2* RNA transcript,
wherein the drug is administered to the
individual under conditions appropriate for entry
15   of the drug into cells in sufficient quantity to
reduce hEST2 protein function or activity.

30. The method of Claim 29 wherein the drug is selected
from the group consisting of: small organic molecules;
enzymes which degrade hEST2 protein; enzyme
20   inhibitors; HEST2 transcriptional regulators;
antisense molecules and dominant negative forms of
hEST2 protein.

ATGAAAATCTTATTCGAGTTCATTCAAGACAAGCTTGACATTGATCTACAGACCAACAGTACTTACAAAGAGAAAATTAAAATGTGGTCACTTCAATGGCC
 M   K   I   L   F   E   F   I   Q   D   K   L   D   I   D   L   Q   T   N   S   T   Y   K   E   N   L   K   C   G   H   F   N   G

TCGATGAAATTCTAACTACGTGTTTCGCACTACCAAATTCAGAAAAAATAGCATTACCATGCCTTCCTGGTGACTTAAGCCACAAAGCAGTCATTGATCA
 L   D   E   I   L   T   C   F   A   L   P   N   S   R   K   I   A   L   P   C   L   P   G   D   L   S   H   K   A   V   I   D   H

CTGCATCATTTACCTGTTGACGGGCGAATTATACAACAACGTACTACAACATTGGCTATAAAATGAAGATGTCAACAATAGTCTTTTTTGC
 C   I   I   Y   L   L   T   G   E   L   Y   N   N   V   L   T   F   G   Y   K   I   A   R   N   E   D   V   N   N   S   L   F   C

CATTCTGCAAATGTTAACGTTACTTTGAAAGGCGCTGCTTGGAAAATGTTCCACAGTTTGGTCGGTACATACGCATTCGTTGATTTATTGATCAATT
 H   S   A   N   V   N   V   T   L   L   K   G   A   A   W   K   M   F   H   S   L   V   G   T   Y   A   F   V   D   L   L   I   N

ATACAGTAATTCAATTTAATGGCCAGTTTTTTCACTCAAATCGTGGGTAACAGATGTAACGAACCTCATCTGCCGCCAAATGGGTCCAACGATCATCCTC
 Y   T   V   I   Q   F   N   G   Q   F   F   T   Q   I   V   G   N   R   C   N   E   P   H   L   P   P   K   W   V   Q   R   S   S   S

ATCATCCGCAACTGCTGCGCAAATCAACAACTTACAGACAACCAGTGACAAATAAACAATTCTTACACAAGCTCAATATAAATTCCTCTCTTTTTTTCCT
 S   S   A   T   A   A   Q   I   K   Q   L   T   E   P   V   T   N   K   Q   F   L   H   L   N   I   N   S   S   F   F   P

TATAGCAAGATCCTTCCTTCATCATCATCTCAAAAAAGTAACTGACTTGAGAGAAGCTATTTTTCCCACAAATTTGGTTAAAATTCCTCAGAGACTAA
 Y   S   K   I   L   P   S   S   S   I   K   K   L   T   D   L   R   E   A   I   F   P   T   N   L   V   K   I   P   Q   R   L

AGGTACGAATTAATTTGACGCTGCAAAGCTATTAAGAGACATAAGCGTTTGAATTACGTTTCTATTTTGAATAGTAATTGCCCACCATTGGAAGGGAC
 K   V   R   I   N   L   T   L   Q   K   L   L   K   R   H   K   R   L   N   Y   V   S   I   L   N   S   I   C   P   P   L   E   G   T

CGTATTGGACTTGTCGCATTTGAGTAGGCAATCACCAAGGAACGAGTCTTGAAATTTATCATTGTGAAATTTTACAGAAGTTATTACCCCAAGAAAATGTTT
 V   L   D   L   S   H   L   S   R   Q   S   P   K   E   R   V   L   K   F   I   I   V   I   L   Q   K   L   L   P   Q   B   M   F

GGCTCCAAGAAAAATAAAGAAAAATTATCAAGAAAATCTTTATTATTAAGTTTTACCCTTAAATGGCTATTTGATAGTTTGTTGTTGAAAAAGT
 G   S   K   K   N   K   G   K   I   I   K   N   L   N   L   L   L   S   L   P   L   N   G   Y   L   P   F   D   S   L   L   K   K

TAAGATTAAAGGATTTTCGGTGGTTGTTCATTTCTGTTCTGATATTTGGTTTTTTTTTTACTGCACCGAAATATCTTCTACAGTGACAATTGTTTACTTTAGACATGATACTTGG
 L   R   L   K   D   F   R   W   L   F   I   S   D   I   W   F   T   K   H   N   F   E   N   L   N   Q   L   A   I   C   F   I   S   W

GCTATTTAGACAACTAATTCCCAAATTATACAGACTTTTTTTTTTTACTGCACCGAAATATCTTCTACAGTGACAATTGTTTACTTTAGACATGATACTTGG
 A   L   L   R   Q   L   I   P   K   I   I   Q   T   F   F   Y   C   T   E   I   S   S   T   V   T   I   V   Y   F   R   H   D   T   W

AATAAACTTATCACCCCCTTTATCGTGTAGAATATTTTTAAGCGTACTTAGTCGAAAACAACGTATGTAGAAACCATAATAATGTCTGTCCAATTTCA
 L   F   R   Q   L   I   P   K   I   I   Q   T   F   F   Y   C   T   E   I   S   S   T   V   T   I   V   Y   F   R   H   D   T   W

AATATAGCAAATGAGGATTATACCAAAAAAGTAATAATGAGTTCAGGATTTTCAGGAATTATACCAGAGGGGCAGAAGAAGAATTCACAATTTA
 N   K   L   I   T   P   F   I   V   E   Y   F   K   T   Y   L   V   E   N   N   V   C   R   N   H   N   S   Y   T   L   S   N   F

ATCATAGCAAATGAGGATTATACCAAAAAAGTAATAATGAGTTCAGGATTTTCAGGAATTATACCAGAGGGGCAGAAGAAGAATTCACAATTTA
 N   H   S   K   M   R   I   I   P   K   K   S   N   E   F   R   I   I   A   I   P   C   R   G   A   D   E   E   F   T   I   Y

TAAGGAGAATCACAAAAATGCTATCCAGCCCACTCAAAAAATTTAGAATACTTGAATACTTGAACAAAGGCCGACTAGTTTTACTAAAATATATTCTCCAACG
 K   E   N   H   K   N   A   I   Q   P   T   Q   K   I   L   E   Y   L   R   N   K   R   P   T   S   F   T   K   I   Y   S   P   T

CAAAATAGCTGACCGTATCAAAGAATTTAAGCAGAGACTTTTAAAGAAATTAATTAAAAATTAATGAATGGGTTTTTCGTTAGATCTCAATATTCTTCAATGTCTCAAATCTT
 K   E   N   H   K   N   A   I   Q   P   T   Q   K   I   L   E   Y   L   R   N   K   R   P   T   S   F   T   K   I   Y   S   P   T

GCTATGATTCCATACCAAGGATGAATGTATGAGAGATTCTGAAGATGCGCTAAAAAATGGGTTTTTCGTTAGATCTCAATATTCTTCAATAC
 Q   I   A   D   R   I   K   E   F   F   K   Q   R   L   L   K   F   N   V   L   P   E   L   Y   F   M   K   F   D   V   K   S

CAATACAGGTGTTGAAGTTATTTAATGTTGTTAACGCTAGCAGAGTACCAAAAACCTTATGAGCTATACATAGATAATGTGAGGGACGGTTCATTTATCA
 C   Y   D   S   I   P   R   M   E   C   M   R   I   L   K   D   A   L   K   N   E   N   G   F   F   V   R   S   Q   Y   F   N   T

AATCAGGATGTTTATAAAACGTTGTAGAGAGATGGAAATAATTAAAACAGCTTTGTGGGTTGAAGATAAGTGCTACATTAGAGAAGATGGTCTTTTTCAGGGCT
 N   T   G   V   L   K   L   F   N   V   V   N   A   S   R   V   P   K   P   Y   E   L   Y   I   D   N   V   R   T   V   H   L   S

 N   Q   D   V   I   N   V   V   E   M   E   I   F   K   T   A   L   W   V   E   D   K   C   Y   I   R   E   D   G   L   F   Q   G

FIG. 1A

CTAGTTTATCTGCTCCGATCGTTGATTTGGTGTATGACGATCTTCTCTGGAGTTTATAGCGAGTTTAAAGCCAGTCCTAGCCAGGACACATTAATTTTAAA
S S L S A P I V D L V Y D D L L E F Y S E F K A S P S Q D T L I L K

ACTGGCTGACGATTCCTTATAATATCAACAGACCAACAGCAAGTGATCAATATCAAAAAGCTTGCCATGGGCGATTCAAAAATATAATGCGAAAGCC
L A D D F L I I S T D Q Q V I N I K K L A M G G F Q K Y N A K A

AATAGAGACAAAATTTAGCCGTAAGCTCCCAATCAGATGATGACGGTTATCAATTTGTGTCAATGCACATATTTGTTAAAGAATTGGAAGTTTGGA
N R D K I L A V S S Q S D D T V I Q F C A M H I F V K E L E V W

AACATTCAAGCACAACAATGAATAATTTCCATATCCGTTCGAAATCTAGTAAAGGGATAATTTCGAAGTTTAACTAGAATCTCTTATAA
K H S S T M N N F H I R S K S S K G I F R S L I A L F N T R I S Y K

AACAATTGACACAAATTTAAATTCAACAAACACCGTTCTCATGCAAATTGATCATGTTGTAAAGAACATTTCGGAATGTTATAAATCTGCTTTTAAGGAT
T I D T N L N S T N T V L M Q I D H V V K N I S B C Y K S A F K D

CTATCAATTAATGTTACGCAAAATATGCAATTTCATTCGTTCTTACAACGCATCATTGAAATGACAGTCAGCGGTTGTCCAATTACGAAATGTGATCCTT
L S I N V T Q N M Q F H S F L Q R I E M T V S G C P I T K C D P

TAATCGAGTATGAGGTACGATTCACCATATTGAATGATTTTGGAAGCCTATCTTCAAACACATCAAAATTTAAAGATAATATCATTCTTTTGAGAAA
L I E Y E V R F T I L N G F L E S L S S N T S K F K D N I L L R K

GGAAATTCAACACTTGCAAGCATATATATATATATATATATATATACACATATAGTTAATTAG 2655
E I Q H L Q A Y I Y I Y I H I V N

FIG. 1B

FIG. 2A

FIG. 2B

MOTIF 1

hEST2	SRLRFIPK
p123	GKLRLIPK
Est2p	SKMRIIPK
a2-Sc	VRRVEIPK
a1-Sc	MRIVNIPK
motif	p hh h K

FIG. 3A

MOTIF 2

hEST2	LRPIVNMDYVVG
p123	FRPIMTFNKKIV
Est2p	FRIIAIPCRGAD
a2-Sc	FRPLSVGNPREK
a1-Sc	IRPLSVGNPRDK
motif	hRh K/R

FIG. 3B

MOTIF 3

*

hEST2	PELYFVKVDVTGAYDTI
p123	PKLFFATMDIEKCYDSV
Est2p	PELYFMKFDVKSCYDSI
a2-Sc	YCNWFIKVDLNKCFDTI
a1-Sc	GSNWFREVDLKKCFDTI
motif	h hDh AF/GY h

FIG. 3C

MOTIF 4

hEST2	KSIVQCGIPQGSILSTLLCSLCY
p123	KFVKQTKGIPQGLCVSSILSSFYV
Est2p	KQVIREDGLFQGSSLSAPIVDLVY
a2-Sc	NYHNTTLGIPQGSVVSPILCNIFL
a1-Sc	TYHKPILGLPQGSLISPILCNIVI
motif	hPQG hP hh h

FIG. 3D

MOTIF 5

**

hEST2	LLLRLMDDFLLVT
p123	LLMRLTDDYLLIT
Est2p	LILKLADDFLIIS
a2-Sc	YFVRYADDIIIGV
a1-Sc	KYVRYADDIIIGV
motif	h Y DDhhh

FIG. 3E

MOTIF 6

hEST2	GCVVNLRKTVV
p123	GFKFNMKKLQT
Est2p	GFQKYNAK--
a2-Sc	GMSINIDKSVI
a1-Sc	GLTINEEKTLI
motif	Gh h cK h

FIG. 3F

FIG. 4

FIG. 5A

2080
GGAGACCAGCCCGCTGAGGGATGCCGTCGTCATCGAGCAGAGCTCCTCCCTGAATGAGGCCAGCAGTGGCCTCTTCGACG   2480
TCTTCCTACGCTTCATGTGCCACCACGCCGTGCGCATCAGGGGCAAGTCCTACGTCCAGTGCCAGGGATCCCGCAGGGC   2560
TCCATCCTCTCCACGCTGCTCTGCAGCCTGCTACGGCGACATGGAGAACAAGCTGTTTGCGGGATTCGGCGGGACGG   2640
GCTGCTCCTGCGTTTGGTGATGATTTCTTGGTGACACCTCACCTCACGCGAAAACCTTCCTCAGGACCCTGG   2720
TCCGAGGTGTCCCCTGAGTATGCCGTCGTGGTGAACTTGCGGTGGTGAACTTGCGGAAGACAGTGGTGAACTTCCCTGTAGAAGACGAGGCCCTG   2800

2880
GGTGGCACGGCTTTGTTGTTCAGATGCCGGCCCACGCCTATTCCCCTGGTGCGGCCTGCTGTGGATACCCGGACCCTGGA   2880
GGTGCAGAGCGACTACTCCAGCTATGCCCGGACCTCCATCAGAGCCAGTCAGTCTCACCTTCAACCGGCTTCAAGGCTGGGA   2960
GGAACATGCGTCGCAAACTCTTTGCGGCTGAAGTGTCACAGCCTGTTTCTGATTTGCAGGTGAACAGCCTC   3040
CAGACGGGTGTGCACCAACATCTACAAGATCCTCCTGCTGCAGGCGTACAGGTTTCACGCATGTGTGCAGCTCCCATT   3120
TCATCAGCAAGTTTGGAAGAACCCCACATTTTTCCTGCGCGTCATCTCTGACACGGCCTCCCCTCGTACTCCATCCTGA   3200

3280
AAGCCAAGAACGCAGGGATGTCTGGGGGCCAAGGGCGCGCCCCTTCTTGCCCTGCCGTGCAGTGGCTGTGC   3280
CACCAAGCATTCCTGCTCAAGCTGACTCGACACCGTGTCACCTACGTGCCCTGGAGGCCTCCTGGGGTCACTCAGGACAGCCCAGAC   3360
GCAGCTGAGTCGGAAGCTCCCGGGACGACGCTGACTGCCCTGAGGCCAACCCGGCCACTGCCCTCAGACTTCA   3440
AGACCATCTGACTGATGGCCACCCGCCCACAGCCCAGAGCAGCACCAGCCCTGTCACGCGGGCTCTAC   3520
GTCCCAGGGAGGGAGGGGCGGCCCCACACCCAGCCCCGCACCCGCTGGGAGTCTGGGAGTTCTGAGGCTGAGTGAGTGTTTGGCCGAGGC   3600

3680
CTGCATGTCCGGCTGAAGGCTGAGTGTCCGGCTGAGGCGAGTGTCCAGCCAAGGGCTCAGTGTCCAGCACACCT   3680
GCCGTCTTCACTTCCCCACAGGCTGGCGCTTCGGCTTCACCCCAGGGCCAGCTTTTCCTCACCAGGAGCCCGGCTTCCACT   3760
CCCCACATAGGAATAGTCCATCCCCAGATTCGCCATTGTTCACCCCTGCCCTCCCTTTGCCTTCACCCCCACCA   3840
TCCAGGTGGAGACCCTGAGAAGGACCTCTGGGAGTCTGGGAATTGGACTGACCAAAGGTGTGCCCTGTACACAGGCGAG   3920
GACCCTGCACCTGGAGGGGGTCCCCTGTGTGGTCAAATTGGGGGAGGTGCGTGTGGGAGTAAAATACTGAATATATGAGTT   4000

4080
TTTCAGTTTTGAAAAAAAAAAAA 4023

FIG. 5B

MPRAPRCRAVRSLLRSHYREVLPLATFVRRLGPQGWRLVQRGDPAAFRALVAQCLVCVPWDARPPPAAPSFR
QVSCLKEL

VARVLQRLCERGAKNVLAFGFALLDGARGPPEAFTTSVRSYLPNTVTDALRGSGAWGLLLRRVGDDVLVH
LLARCALFV

LVAPSCAYQVCGPPLYQLGAATQARPPPHASGPRRRLGCERAWNHSVREAGVPLGLPAPGARRRGGSASRSL
PLPKRPRR

GAAPEPERTPVGGQGSWAHPGRTRGPSDRGFCVVSPARPAEEATSLEGALSGTRHSHPSVGRQHHAGPPSTSRPP
RPWDTP

CPPVYAETKHFLYSSGDKEQLRPSFLLSSLRPSLTGARRLVETIFLGSRPWMPGTPRRLPRLPQRYWQMRPLFL
ELLGNH

AQCPYGVLLKTHCPLRAAVTPAAGVCAREKPQGSVAAPEEDTDPRRLVQLLRQHSSPWQVYGFVRACLRRL
VPPGLWGS

RHNERRFLRNTKKFISLGKHAKLSLQELTWKMSVRGCAWLRRSPGVGCVPAAEHRLREEILAKFLHWLMSVY
VVELLRSF

FYVTETTFQKNRLFFYRKSVWSKLQSIGIRQHLKRVQLRELSEAEVRQHREARPALLTSRLRFIPKPDGLRPIVN
MDYVV

GARTFRREKRAERLTSRVKALFSVLNYERARRPGLLGASVLGLDDIHRAWRTFVLRVRAQDPPPELYFVKVD
VTGAYDTI

PQDRLTEVIASIIKPQNTYCVRRYAVVQKAAHGHVRKAFKSHVSTLTDLQPYMRQFVAHLQETSPLRDAVVIE
QSSSLNE

ASSGLFDVFLRFMCHHAVRIRGKSYVQCQGIPQGSILSTLLCSLCYGDMENKLFAGIRRDGLLLRLVDDFLLVT
PHLTHA

KTFLRTLVRGVPEYGCVVNLRKTVVNFPVEDEALGGTAFVQMPAHGLFPWCGLLLDTRTLEVQSDYSSYART
SIRASLTF

NRGFKAGRNMRRKLFGVLRLKCHSLFLDLQVNSLQTVCTNIYKILLQAYRFHACVLQLPFHQQVWKNPTFF
LRVISDTA

SLCYSILKAKNAGMSLGAKGAAGPLPSEAVQWLCHQAFLLKLTRHRVTYVPLLGSLRTAQTQLSRKLPGTTLT
ALEAAAN
PALPSDFKTILD

FIG. 6

10/14

```
        10        20        30        40
GTCTCACTCTGTCACCCAGGCTGGAGTGTAATGGCACAAT    40
CTCGGCTCACTGCAACCTCTGCCTCCTCGGTTCAAGCAGT    80
TCTCATTCCTCAACCTCATGAGTAGCTGGGATTACAGGCG   120
CCCACCACCACGCCTGGTTAATTTTTGTATTTTTAGTAGA   160
GATAGGCTTTCACCATGTTGGCCAGGCTGGTCTCAAACTC   200


        210       220       230       240
CTGACCTCAAGTGATCTGCCCGCCTTGGCCTCCCACAGTG   240
CTGGGATTACAGGTGCAAGCCACCGTGCCCGGCATACCTT   280
GATCTTTTAAAATGAAGTCTGAAACATTGCTACCCTTGTC   320
CTGAGCAATAAGACCCTTAGTGTATTTAGCTCTGGCCAC   360
CCCCCAGCCTGTGTGCTGTTTTCCTGCTGACTTAGTTCTA   400


        410       420       430       440
TCTCAGGCATCTTGACACCCCCACAAGCTAAGCATTATTA   440
ATATTGTTTTCCGTGTTGAGTGTTTCTGTAGCTTTGCCCC   480
CGCCCTGCTTTTCCTCCTTTGTTCCCCGTCTGTCTTCTGT   520
CTCAGGCCCGCCGTCTGGGGTCCCCTTCCTTGTCCTTTGC   560
GTGGTTCTTCTGTCTTGTTATTGCTGGTAAACCCCAGCTT   600


        610       620       630       640
TACCTGTGCTGGCCTCCATGGCATCTAGCGACGTCCGGGG   640
ACCTCTGCTTATGATGCACAGATGAAGATGTGGAGACTCA   680
CGAGGAGGGCGGTCATCTTGGCCCGTGAGTGTCTGGAGCA   720
CCACGTGGCCAGCGTTCCTTAGCCAGTGAGTGACAGCAAC   760
GTCCGCTCGGCCTGGGTTCAGCCTGGAAAACCCCAGGCAN   800


        810       820       830       840
GTCGGGGTCTGGTGGCTCCGCGGTGTCGAGTTTGAAATCG   840
CGCAAACCTGCGGTGTGGCGCCAGCTCTGACGGTGCTGCC   880
TGGCGGGGGAGNGTCTGCTTCCTCCCTTCTGCTTGGGAAC   920
CAGGACAAAGGATGAGGCTCCGAGCCGTTGTCGCCCAACA   960
GGAGCATGACGGGTTGGCTGTGTTCCGGCCGCAGAGCACC  1000


       1010      1020      1030      1040
GTCTGCGTGAGGAGATCCTGGCCAAGTTCCTGCACTGGCT  1040
GATGAGTGTGTACGTCGTCGAGCTGCTCAGGTCTTTCTTT  1080
TATGTCACGGAGACCACGTTTCAAAAGAACAGGCTCTTTT  1120
TCTACCGGAAGAGTGTCTGGAGCAAGTTGCAAAGCATTGG  1160
AATCAGACAGCACTTGAAGAGGGTGCAGCTGCGGGAGCTG  1200
```

FIG. 7A

	1210	1220	1230	1240	
TCGGAAGCAGAGGTCAGGCAGCATCGGGAAGCCAGGCCCG					1240
CCCTGCTGACGTCCAGACTCCGCTTCATCCCCAAGCCTGA					1280
CGGGCTGCGGCCGATTGTGAACATGGACTACGTCGTGGGA					1320
GCCAGAACGTTCCGCAGAGAAAAGAGGGCCGAGCGTCTCA					1360
CCTCGAGGGTGAAGGCACTGTTCAGCGTGCTCAACTACGA					1400

	1410	1420	1430	1440	
GCGGGCGCGGCGCCCCGGCCTCCTGGGCGCCTCTGTGCTG					1440
GGCCTGGACGATATCCACAGGGCCTGGCGCACCTTCGTGC					1480
TGCGTGTGCGGGCCCAGGACCCGCCGCCTGAGCTGTACTT					1520
TGTCAAGGTGGATGTGACGGGCGCGTACGACACCATCCCC					1560
CAGGACAGGCTCACGGAGGTCATCGCCAGCATCATCAAAC					1600

	1610	1620	1630	1640	
CCCAGAACACGTACTGCGTGCGTCGGTATGCCGTGGTCCA					1640
GAAGGCCGCCCATGGGCACGTCCGCAAGGCCTTCAAGAGC					1680
CACGTCCTACGTCCAGTGCCAGGGGATCCCGCAGGGCTCC					1720
ATCCTCTCCACGCTGCTCTGCAGCCTGTGCTACGGCGACA					1760
TGGAGAACAAGCTGTTTGCGGGGATTCGGCGGGACGGGCT					1800

	1810	1820	1830	1840	
GCTCCTGCGTTTGGTGGATGATTTCTTGTTGGTGACACCT					1840
CACCTCACCCACGCGAAAAACCTTCCTCAGGACCCTGGTCC					1880
GAGGTGTCCCTGAGTATGGCTGCGTGGTGAACTTGCGGAA					1920
GACAGTGGTGAACTTCCCTGTAGAAGACGAGGCCCTGGGT					1960
GGCACGGCTTTTGTTCAGATGCCGGCCCACGGCCTATTCC					2000

	2010	2020	2030	2040	
CCTGGTGCGGCCTGCTGCTGGATACCCGGACCCTGGAGGT					2040
GCAGAGCGACTACTCCAGCTATGCCCGGACCTCCATCAGA					2080
GCCAGTCTCACCTTCAACCGCGGCTTCAAGGCTGGGAGGA					2120
ACATGCGTCGCAAACTCTTTGGGGTCTTGCGGCTGAAGTG					2160
TCACAGCCTGTTTCTGGATTTGCAGGTGAACAGCCTCCAG					2200

	2210	2220	2230	2240	
ACGGTGTGCACCAACATCTACAAGATCCTCCTGCTGCAGG					2240
CGTACAGGTTTCACGCATGTGTGCTGCAGCTCCCATTTCA					2280
TCAGCAAGTTTGGAAGAACCCCACATTTTTCCTGCGCGTC					2320
ATCTCTGACACGGCCTCCCTCTGCTACTCCATCCTGAAAG					2360
CCAAGAACGCAGCCGAAGAAAACATTTCTGTCGTGACTCC					2400

	2410	2420	2430	2440	
TGCGGTGCTTGGGTCGGGACAGCCAGAGATGGAGCCACCC					2440
CGCAGACCGTCGGGTGTGGGCAGCTTTCCGGTGTCTCCTG					2480
GGAGGGGAGCTGGGCTGGGCCTGTGACTCCTCAGCCTCTG					2520
TTTTCCCCCAGGGATGTCGCTGGGGGCCAAGGGCGCCGCC					2560
GGCCCTCTGCCCTCCGAGGCCGTGCAGTGGCTGTGCCACC					2600

FIG. 7B

```
            2610      2620      2630      2640
AAGCATTCCTGCTCAAGCTGACTCGACACCGTGTCACCTA      2640
CGTGCCACTCCTGGGGTCACTCAGGACAGCCCAGACGCAG      2680
CTGAGTCGGAAGCTCCCGGGGACGACGCTGACTGCCCTGG      2720
AGGCCGCAGCCAACCCGGCACTGCCCTCAGACTTCAAGAC      2760
CATCCTGGACTGATGGCCACCCGCCCACAGCCAGGCCGAG      2800
```

```
            2810      2820      2830      2840
AGCAGACACCAGCAGCCCTGTCACGCCGGGCTCTACGTCC      2840
CAGGGAGGGAGGGGCGGCCCACACCCAGGCCCGCACCGCT      2880
GGGAGTCTGAGGCCTGAGTGAGTGTTTGGCCGAGGCCTGC      2920
ATGTCCGGCTGAAGGCTGAGTGTCCGGCTGAGGCCTGAGC      2960
GAGTGTCCAGCCAAGGGCTGAGTGTCCAGCACACCTGCCG      3000
```

```
            3010      3020      3030      3040
TCTTCACTTCCCCACAGGCTGGCGCTCGGCTCCACCCCAG      3040
GGCCAGCTTTTCCTCACCAGGAGCCCGGCTTCCACTCCCC      3080
ACATAGGAATAGTCCATCCCCAGATTCGCCATTGTTCACC      3120
CCTCGCCCTGCCCTCCTTTGCCTTCCACCCCCACCATCCA      3160
GGTGGAGACCCTGAGAAGGACCCTGGGAGCTCTGGGAATT      3200
```

```
            3210      3220      3230      3240
TGGAGTGACCAAAGGTGTGCCCTGTACACAGGCGAGGACC      3240
CTGCACCTGGATGGGGGTCCCTGTGGGTCAAATTGGGGGG      3280
AGGTGCTGTGGGAGTAAAATACTGAATATATGAGTTTTTC      3320
AGTTTTGAAAAAAAAAAAAGGAATTC 3346
```

FIG. 7C

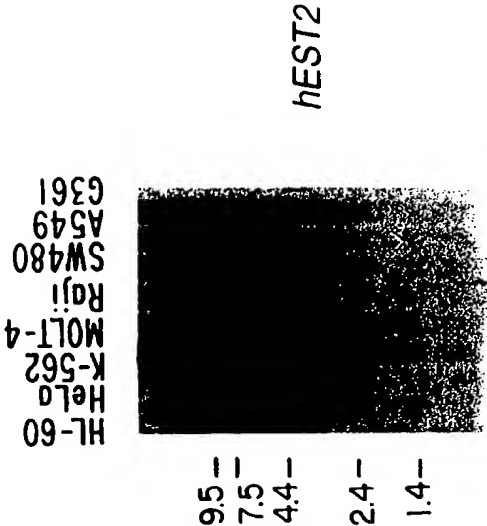FIG.8A FIG.8B FIG.8C

Normal HumanTissues

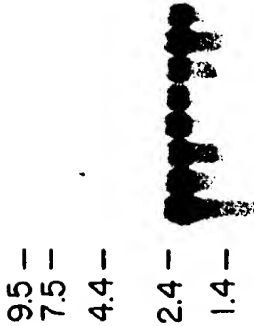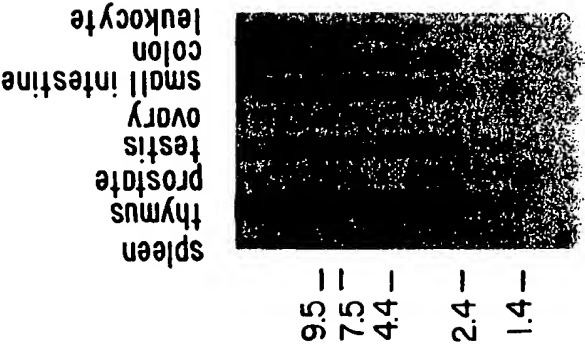Cancer Cell Lines

hEST2

actin

FIG.8D FIG.8E FIG.8F

FIG.9B

FIG.9A

FIG.9D

FIG.9C